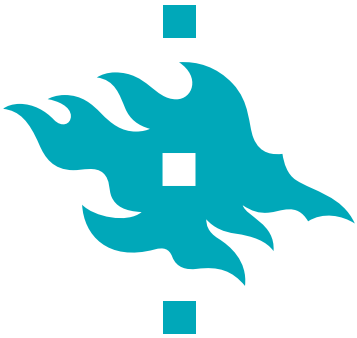

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

PETRA MARIA PÖLLÄNEN

DYNAMICS OF THE DISEASE PROCESS LEADING TO TYPE 1 DIABETES IN CHILDREN WITH HLA-CONFERRED DISEASE SUSCEPTIBILITY

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Dynamics of the disease process leading to type 1 diabetes in children with HLA-conferred disease susceptibility

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ACADEMIC DISSERTATION

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ABSTRACT

Background

Type 1 diabetes is an immune-mediated endocrine disorder that affects approximately 0.8% of children and adolescents in Finland, which has the highest incidence of type 1 diabetes in the world. The burden of type 1 diabetes on affected individuals, their families, and the healthcare system is notable, and the increasing incidence of type 1 diabetes worldwide calls for preventive measures. The disease process is driven by progressive destruction of pancreatic insulin-producing beta cells, eventually leading to total insulin deficiency and causing symptoms of severe hyperglycemia. The etiology of type 1 diabetes is considered to be highly multifactorial, and both genetic and environmental factors are likely to contribute to the disease pathogenesis. A presymptomatic period of highly variable duration precedes the onset of clinical type 1 diabetes, during which autoantibodies to multiple beta-cell antigens are detected in the circulation of prediabetic individuals. Together with human leukocyte antigen (HLA) genotypes, the autoantibodies may be used for disease prediction. However, the factors decisive for individual disease risk and the progression rate to clinical type 1 diabetes have thus far remained incompletely understood. The highly variable duration of the preclinical period suggests that the genetic, immunological, epigenetic, and/or environmental factors substantially affect the pace of progression to clinical disease.

Aims

This thesis aims at improving the early prediction of clinical type 1 diabetes and the timing of disease onset by characterizing the genetic, immunological, and demographic factors associated with the progression rate to type 1 diabetes and by describing the predictive islet autoantibody dynamics during the first 15 years in HLA-predisposed children.

Subjects and methods

The study subjects in this thesis are participants in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study. The prospective DIPP study aims at monitoring the development of islet autoimmunity and type 1 diabetes in HLA-predisposed children and at identifying means for delaying or preventing the onset of clinical diabetes. Newborn infants born in the Turku, Oulu, and Tampere University Hospitals, Finland, are screened for HLA genotypes predisposing to type 1 diabetes from cord blood. Eligible infants are invited to clinical follow-up, including islet autoantibody assessment starting from the age of 3 months. Islet cell antibodies (ICA) and autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), islet antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A) are analyzed as markers of islet autoimmunity from venous blood samples obtained at clinical visits every 3–12 months.

To assess the dynamics of islet autoantibodies in childhood, the first 1006 DIPP children (53.0% boys) born in 1994–1997 were followed from birth up to 15 years of age (Study I). In this study, ZnT8A had been analyzed from all samples available. In Studies II and III, a population of 7410 DIPP children (52.6% boys) was followed from birth for a median time of 16.2 years in order to identify genetic, immunological, and demographic characteristics of rapid and slow progression to type 1 diabetes. Multiple non-HLA single-nucleotide polymorphisms (SNPs) predisposing to type 1 diabetes were analyzed for potential associations with the progression rate. No data on ZnT8A were available for the study population in Studies II and III.

Results

Among the 1006 DIPP children, the ICA seroconversion rate increased during the first 15 years, but the biochemical autoantibodies showed age-dependent decreasing seroconversion rates. In young children, IAA and ZnT8A appeared commonly as the primary autoantibodies, but in preschool years, IA-2A and especially GADA-initiated autoimmunity became common. One frequent phenomenon, loss of IAA positivity, indicated delayed progression from seroconversion to diagnosis compared with steady IAA positivity (median delay 8.2 vs. 3.4 years; P=0.01).

Among the 7410 DIPP children, 42 (16.9% of progressors) progressed to type 1 diabetes rapidly, within 1.5 years from seroconversion. Relative to slower progressors, the rapid progressors had a higher frequency of positivity for multiple (≥2) autoantibodies, higher titers of ICA, IAA, and IA-2A at seroconversion, and a higher prevalence of the homozygous *FUT2* secretor genotype and the high-risk *HLA-DQB1*02/*03:02* genotype. Rapid progression occurred in both young (age <5 years) and early pubertal children (age >7 years), resulting in a double-peak profile in seroconversion age. The young rapid progressors were characterized by IAA positivity and high IAA titers at seroconversion, while in the older subgroup GADA positivity and high GADA titers were frequent. Slow progressors were distinguished from other progressors by lower titers of ICA and IAA, and lower rate of positivity for IA-2A and multiple autoantibodies at seroconversion. Appearance of multiple autoantibodies was delayed among slow progressors. Season of birth differed between slow and other progressors. Slow progressors (n=62, 25.1%) were born more often in the fall than other progressors (31% vs. 22%), while the other progressors tended to be born more often in the spring (31% vs. 15%). No significant differences emerged in the non-HLA SNP distributions between slow and other progressors after correction for multiple testing.

Conclusions

This thesis presents a detailed prospective description of the dynamics of islet autoantibodies during the first 15 years in HLA-susceptible children from the Finnish pediatric population. It also provides the first combined characterization of genetic, immunological, and demographic factors associated with both rapid and slow progression from seroconversion to type 1 diabetes. This was the first study to systematically assess the role of ZnT8A in the context of preclinical autoantibody testing used for prediction of type 1 diabetes.

Novel findings in this thesis were the double-peak profile of seroconversion age among rapid progressors to type 1 diabetes, the variation in the seasonality of birth among progressors to type 1 diabetes, the association of the *FUT2* SNP with rapid progression, and the early appearance of ZnT8A in young children. This study demonstrated that rapid progressors differ from slower progressors by genetic, immunological, and demographic factors present at seroconversion. Depending on age, the immunological characteristics of rapid progressors are diverse, suggesting that triggers of aggressive islet autoimmunity may be heterogeneous.

As expected, the primary islet autoantibody turned out to be highly characteristic of age. This highlights the existence of more than one endotype of islet autoimmunity and suggests that age and the stage of immunological maturation contribute substantially to the characteristics of beta-cell autoimmunity. The stability of islet autoantibodies might affect the risk for type 1 diabetes. The findings in this thesis improve the estimation of preclinical diabetes risk and the timing of disease onset, providing a clinically valuable framework for individualizing the efforts to delay or prevent type 1 diabetes. As soon as a safe and reliable preventive measure is available, the discoveries now made can be utilized to apply these methods to suitable populations at risk.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Pöllänen PM, Ryhänen SJ, Toppari J, Ilonen J, Vähäsalo P, Veijola R, Siljander H, Knip M. Dynamics of islet autoantibodies during prospective follow-up from birth to age 15 years. *J Clin Endocrinol Metab* 2020;105:dgaa624. doi: 10.1210/clinem/dgaa624

II Pöllänen PM, Lempainen J, Laine AP, Toppari J, Veijola R, Vähäsalo P, Ilonen J, Siljander H, Knip M. Characterisation of rapid progressors to type 1 diabetes among children with HLA-conferred disease susceptibility. *Diabetologia* 2017;60:1284–1293

III Pöllänen PM, Lempainen J, Laine AP, Toppari J, Veijola R, Ilonen J, Siljander H, Knip M. Characteristics of slow progression to type 1 diabetes in children with increased HLA-conferred disease risk. *J Clin Endocrinol Metab* 2019;104:5585–5594

The publications are referred to in the text by their Roman numerals and have been reprinted with the permission of their respective copyright holders. In addition, some unpublished material is presented.

ABBREVIATIONS

ABCD	Autoimmune Beta Cell Disorder
AIDA	Anti-Interleukin-1 in Diabetes Action
AIRE	Autoimmune regulator
APC	Antigen-presenting cell
BCR	B-cell receptor
BMI	Body mass index
CD	Cluster of Differentiation; attached number indicates the cluster, e.g. CD4
CI	Confidence interval
COVID-19	Coronavirus disease 2019
CXCR5	C-X-C chemokine receptor type 5
DAISY	Diabetes Autoimmunity Study in the Young
DASP	Diabetes Autoantibody Standardization Program
DEFEND-1	Durable Response Therapy Evaluation For Early or New-Onset Type 1 Diabetes
DENIS	Deutsche Nicotinamide Intervention Study
DIAPREV-IT	Diabetes Prevention – Immune Tolerance
DIPP	Type 1 Diabetes Prediction and Prevention
DPT-1	Diabetes Prevention Trial—Type 1
EBI2	Ebstein-Barr virus-induced G protein coupled receptor
ENDIT	European Nicotinamide Diabetes Intervention Trial
ER	Endoplasmic reticulum
FDR	First-degree relative
FOXP3	Forkhead box P3
FPIR	First-phase insulin response
FUT2	1,2-α-Fucosyltransferase
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GADA	Autoantibodies to glutamic acid decarboxylase
GPR183	G-protein coupled receptor 183
GWAS	Genome-wide association study
HbA1c	Glycated hemoglobin
HLA	Human leukocyte antigen
HR	Hazard ratio
IAA	Autoantibodies to insulin
IASP	Islet Autoantibody Standardization Program
IA-2	Islet antigen-2
IA-2A	Autoantibodies to islet antigen-2
IA-2β	Islet antigen-2 beta
ICA	Islet cell antibodies
IDDM	Insulin-dependent diabetes mellitus
IFN-γ	Interferon-γ
Ig	Immunoglobulin; attached letter indicates the subtype, e.g. IgA

IGF	Insulin-like growth factor; attached number indicates the subtype, e.g. IGF1
IGT	Impaired glucose tolerance
IKZF4	IKAROS family zinc finger 4
IL-2	Interleukin-2
IL-6	Interleukin-6
INIT-II	Intranasal Insulin Trial II
INS	Insulin gene
JDFU	Juvenile Diabetes Foundation Unit
JDRF	Juvenile Diabetes Research Foundation
LD	Linkage disequilibrium
LYP	Lymphoid tyrosine phosphatase
NA	Not available
NOD	Non-obese diabetic
NS	Non-significant
OGTT	Oral glucose tolerance test
OR	Odds ratio
P_c	Corrected *P*-value
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PHTF1	Putative homeodomain transcription factor 1
PPV	Positive predictive value
Pre-POINT	Primary Oral Insulin Trial
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RNA	Ribonucleic acid
RU	Relative unit
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SLC30A8	Solute carrier family 30 member 8
SNAIL	Slow or Nonprogressive Autoimmunity to the Islets of Langerhans
SNP	Single-nucleotide polymorphism
T1D	Type 1 diabetes
T1DAL	Inducing Remission in New-Onset Type 1 Diabetes with Alefacept Study
TCR	T-cell receptor
TEDDY	The Environmental Determinants of Diabetes in the Young
Th17	T helper 17
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
UPR	Unfolded protein response
VDJ	VDJ recombination, a mechanism of somatic recombination in developing lymphocytes during which T- or B-cell receptor gene segments are randomly recombined; variable (V), diverse (D), joining (J) segments
VIGR	Viruses in the Genetically at Risk
VNTR	Variable number tandem repeat
ZnT8	Zinc transporter 8
ZnT8A	Autoantibodies to zinc transporter 8

INTRODUCTION

Type 1 diabetes is a chronic autoimmune-mediated endocrine disorder characterized by the progressive loss of insulin-producing beta cells in the pancreas. When a critical proportion of beta cells is destroyed, the patient becomes permanently insulin-deficient and reliant on exogenous insulin treatment.

A rapid rise in the incidence of type 1 diabetes in developed countries over the past decades has created the need for novel means of preventing or delaying the onset of clinical type 1 diabetes (1). The increase in the incidence has been reported to be especially rapid in young children, which has raised suspicions of the role of aggressive autoimmune responses and the failure of self-tolerance mechanisms in children (2). Type 1 diabetes causes an excessive burden on affected individuals and their families and puts a strain on the healthcare system and society. Children with type 1 diabetes have increased mortality and risk of cardiovascular comorbidity compared with the general pediatric population (3, 4). Therefore, it is obvious that studies on the etiology, pathogenesis, and prediction and prevention of type 1 diabetes are urgently needed.

Type 1 diabetes is a heterogeneous disease (5). The disease begins with an asymptomatic period of highly variable duration during which autoantibodies against beta-cell structures usually appear into the circulation of prediabetic individuals and can be interpreted as a sign of ongoing beta-cell damage (6). Towards the advanced stages of the process, disturbances in glucose metabolism become more common as the beta-cell destruction proceeds.

However, not all genetically susceptible children with islet autoantibodies progress to clinical disease (7). At present, the most reliable and attainable strategies to identify individuals at risk for type 1 diabetes are based on screening for type 1 diabetes-associated HLA genotypes and preclinical islet autoantibody profiles (8). Despite substantial research efforts, screening for genetic and autoantibody markers has remained a rather poor tool for predicting the onset of clinical disease if no metabolic assessments are performed. The heterogeneous duration of the prediabetic phase suggests that, in addition to genetic factors, environmental elements modify the pace of the disease progression.

This thesis aims at improving the early prediction of type 1 diabetes and the timing of its onset by characterizing genetic, immunological, and demographic factors associated with both rapid and slow progression to type 1 diabetes and by identifying dynamics of the most common islet autoantibodies during the first 15 years in HLA-predisposed children recruited from the general population.

REVIEW OF THE LITERATURE

HISTORY AND CLASSIFICATION OF DIABETES

Diabetes mellitus comprises a heterogeneous group of metabolic diseases with the common characteristic of hyperglycemia. Depending on the form of diabetes, the hyperglycemia is caused by defective insulin secretion, impaired insulin action on target tissues, or both. The classical symptoms of hyperglycemia include increased urination, thirst, weight loss, and fatigue. Untreated diabetes can lead to acute life-threatening complications, most importantly diabetic ketoacidosis. In long-term diabetes, secondary complications often arise (9). Patients with diabetes have increased risk of cardiovascular diseases and increased mortality (3, 4). Although several forms of diabetes exist, most cases can be classified into one of two main etiopathogenic categories, type 1 or type 2 diabetes (9).

Type 1 diabetes is a chronic endocrine disorder usually diagnosed in children and adolescents, but it can develop at any age. The disease is characterized by immune-mediated destruction of insulin-producing beta cells in the pancreatic islets of Langerhans, eventually resulting in almost total deficiency of endogenous insulin production (9).

Throughout history, type 1 diabetes has been recognized as a condition in which the affected person urinates excessively and loses weight, with the urine having the sweet taste of honey or sugar (10). Descriptively, the term diabetes originated from the Greek word ‘diabetes’, which means ‘to siphon’ or ‘to pass through’, referring to increased urination (11). Before the discovery of exogenous insulin therapy, the life expectancy of a patient with type 1 diabetes was poor, and the disease was generally considered deadly.

In 1889, an observation was made that the removal of the pancreas from dogs caused fatal diabetes, which indicated for the first time the role of the pancreas in diabetes (12). The islets of Langerhans were first discovered already in 1869 by Paul Langerhans, who noted that two types of cell populations exist in the pancreas, the first secreting the regular pancreatic juice and the other having an unidentified function (13). Later, in 1907, these cell clusters of unknown properties were given the name islets of Langerhans, and, eventually in 1921, experiments on pancreatic extracts of dogs resulted in the discovery of insulin and the successful treatment of the first type 1 diabetes patient with insulin (14). The heterogeneity in the etiopathogenesis of diabetes was not, however, recognized until in 1951. The observation that not all patients with diabetes were insulin-deficient led to the categorical discrimination between insulin-dependent type 1 and non-insulin-dependent type 2 diabetes (15). The association of autoimmunity with type 1 diabetes was discovered in the 1970s, when the first diabetes-associated autoantibodies, islet cell antibodies, were described (16). Since then, the discovery of autoimmunity behind type 1 diabetes has paved the way for a series of fundamental advances in the study of the disease pathogenesis, many of which are introduced in the following sections.

EPIDEMIOLOGY OF TYPE 1 DIABETES

Globally, over 1.1 million children and adolescents under the age of 20 years have been diagnosed with type 1 diabetes and the disease incidence continues to rise at an annual rate of 3.4%, calling for

effective preventive measures (1, 17). Type 1 diabetes was a relatively rare disease until the 1950s, after which the incidence started to increase in many countries at the same time (18). The increase in the incidence has been especially rapid among children under the age of 5 years and in countries that previously had a low incidence (19). Finland has the highest incidence of type 1 diabetes in the world (1). Overall, the incidence is high in developed countries, and low in low-income countries with a poor standard of living. The increase in the incidence rate has been similar in boys and girls in the age groups 0–4 and 5–9 years, but in the age group 10–14 years the rate has been higher in boys than in girls (1).

In the 1990s, the incidence of type 1 diabetes in Finland was estimated to increase to 50/100 000 by 2010 (19, 20). However, the highest incidence was observed already in 2006, reaching 65/100 000 new cases per year (21). By the end of 2011, the incidence rate of type 1 diabetes in the Nordic countries had reached a phase of plateau, showing no subsequent increases (1, 21, 22). Interestingly, some studies have reported cyclic variation in the incidence of type 1 diabetes, but the evidence for this phenomenon is inconsistent (1).

The high incidence of type 1 diabetes in young children calls for innovative means for preventing or delaying the onset of the clinical disease. Genetic factors alone do not explain the rapid increase in type 1 diabetes, and thus, environmental etiologies or epigenetic factors are likely to contribute to the disease pathogenesis. This is also evident from studies on type 1 diabetes among monozygotic twins and immigrants (23–25). The decreasing age at the diagnosis of type 1 diabetes has raised questions of whether this is due to more aggressive autoimmune responses, dysregulation of immunological maturation, or defects in the mechanisms sustaining self-tolerance (2).

THE IMMUNE SYSTEM

Innate immunity

The mammalian immune response undergoes three phases in the following order: the immediate innate immune response, the induced innate defenses, and the adaptive immune responses. Accordingly, the innate immune system forms the first line of defense against invading microbial pathogens in the human body.

The primary role of the innate immune system is to localize the site of infection and to keep the infection from spreading until an adaptive immune response is established. The immediate innate immunity comprises relatively simple mechanisms that are identical to any given pathogen and are constantly prepared to fight an invading microbe.

First, the physical and chemical barriers on epithelial and mucosal surfaces resist microbial colonization and invasion through several mechanisms (26). Second, the complement system consists of a range of plasma proteins involved in three pathways of cleavage reactions that facilitate the lysis or phagocytosis of invading pathogens (27).

Innate immune cells express multiple receptor systems that recognize diverse microbial structures. Based on the wide repertoire of receptors, such as pattern recognition receptors, these cells mediate rapid elimination of pathogens through phagocytosis, but induce also longer proinflammatory responses (28). Activation of cytokine production leads to recruitment of immune cells to the infection site, and promotes the initiation of the adaptive immune response by guiding

antigen-presenting cells (APCs) to lymphoid tissues (29). If the innate immune mechanisms fail to eradicate the infection, the recruitment of the adaptive immune system has already started.

Adaptive immunity

Whereas the innate immune response remains identical from one pathogen to another, the adaptive immune system communicates through an exquisite antigen-epitope recognition system and is capable of conducting highly specific and targeted responses to nearly any given pathogen. This ability results from a virtually indefinite repertoire of antigen-specific lymphocyte receptors unique to each lymphocyte and generated through random recombination of receptor gene segments and combinations of variable protein chains; heavy and light chains in the case of immunoglobulins and two chains (mostly α and β chains) in the case of T-cell receptors (TCRs). The adaptive immune response is characterized by high antigen-specificity and sensitivity achieved through the selection and amplification of the most antigen-specific lymphocyte clones in the process of clonal expansion.

Cell-mediated immunity

Cell-mediated immunity comprises adaptive immune responses that require cell-to-cell contact and involve T cells. Humoral immunity refers to immune responses that are mediated through the actions of immunoglobulins, produced by B cells. Naïve T and B cells awaiting activation are strictly organized into particular zones in the lymphoid tissues. This organization is regulated by chemokines and facilitates the interaction of naïve lymphocytes with APCs and with each other (30).

Activation of naïve T lymphocytes requires antigen presentation by APCs in a peptide-human leukocyte antigen (HLA) complex (31). The main APCs are dendritic cells, B lymphocytes, and macrophages. After primary activation, the T cells undergo clonal expansion and evolve into effector cells that are capable of efficiently invading the pathogen. The type of effector function that the T cell gains during clonal expansion is determined by the surrounding cytokine environment. Effector T cells orchestrate virtually the entire entity of effector mechanisms in the innate and adaptive immune response (32, 33). After acquiring effector function, the T cell can react to any cell that presents their cognate antigen independently of costimulation.

The effector functions of T cells are mediated by the actions of cytokines and the binding of membrane-associated effector molecules to their specific receptors. Cytotoxic CD8 T cells release prestored cytotoxins into the target cell and secrete interferon-γ (IFN-γ) that inhibits viral replication. IFN-γ causes hyperexpression of HLA class I molecules on the surface of virus-infected cells (34). Another event mediating function of cytotoxic T cells might be the binding of membrane-bound Fas ligand to Fas on the target cell, which induces apoptosis.

The predominant TCR consists of two protein chains, the TCRα and TCRβ. TCRs are structurally highly variable molecules that are cell surface-bound and unable to bind unprocessed antigens. Instead, they recognize the combination ligand of a peptide-self HLA complex. Each TCR is restricted to a specific peptide-HLA combination. In addition, T cells respond to this complex only if the same APC bears costimulatory molecule B7 on its surface to pair with CD28 molecule on the T cell (35). This double requirement for T-cell activation prevents the naïve T cells from turning autoreactive towards self-tissues, which do not express the required costimulatory molecules.

Two classes of HLA molecules exist, defined by their binding to either costimulatory molecule CD8 or CD4 on the T cell. Accordingly, the two functional classes of α-β T cells are HLA-restricted. Only CD8-positive T cells bind to peptides presented on a peptide-HLA class I complex, while only CD4-positive T cells bind to peptide-HLA class II complex (35).

Humoral immunity

B cells are responsible for the production of antibodies, which function in immune defense via several mechanisms. These include neutralization of pathogens by blocking their ability to bind to host receptors and the activation of the classical pathway of the complement system. Immunoglobulins (Igs) are found in both soluble and membrane-bound forms as B-cell receptors (BCRs) on the B-cell surface.

Several functionally different antibody isotypes exist, namely the classes IgG, IgM, IgA, and IgE. The effector action of a certain isotype is defined by the structure of the Ig Fc region (non-antigen binding site), which binds to Fc receptors on immune cells (36). However, the overall Ig structure is similar for all isotypes. Igs are Y-shaped molecules that comprise two identical heavy chains and two identical light chains. Each chain exhibits a constant (C) region and a variable (V) region, the latter of which contributes to the antigen-binding site. Each Ig molecule contains two antigen-binding sites, which promotes higher avidity of antigen binding and enables cross-linking of antigens (37). The BCR is unique to each B cell, and this variability is achieved through the process of VDJ recombination. Gene segments of the Ig V region are randomly combined to produce a unique nucleotide sequence that can be successfully translated into a protein (38).

Naïve B cells reside in the secondary lymphoid tissues awaiting activation. Although some thymus-independent non-peptide antigens can induce antibody production in the absence of T cells, most Ig production is T-cell-dependent. Therefore, B-cell activation requires binding of the specific antigen to the BCR and additional B-cell interaction with the cognate CD4 helper T cell in a process called linked recognition (39). B cells are APCs and present their antigens to CD4 helper T cells on a peptide-HLA class II complex.

After activation, a proportion of B cells become short-lived plasmablasts, while the rest move to the germinal center of the secondary lymphoid tissue to undergo somatic hypermutation and Ig class switching. In the germinal center reaction, the proliferation and clonal expansion of B cells is controlled by follicular CD4 helper T cells and relies on T-cell stimulation by cytokine release and through the binding of the CD40 ligand (T cells) to CD40 (B cells). During somatic hypermutation, the Ig genes in B cells undergo random point mutations across the Ig V region, resulting in antibody affinity maturation. Consequently, B cells producing the highest affinity antibodies become selected for survival and transform into long-lived antibody-secreting plasma cells or memory B cells. Ig class switching results from recombination of the Ig gene C region, which leads to an increased range of the functional properties of antibodies reacting to the same antigen specificity (40).

During infection the T-cell-dependent Ig production begins with IgM class antibodies, thereafter rapidly spreading to other Ig isotypes. The first-produced IgM class antibodies are of low affinity, but are efficient in the activation of the complement system. Natural antibodies produced in the absence of infection are of IgM class. IgG class antibodies, in turn, exhibit higher affinity to antigens and persist long after acute infection. IgA and IgE are especially important in immune defenses below epithelial body surfaces.

Immunological memory

A unique feature of the adaptive immune system is immunological memory against encountered and successfully eradicated pathogens. After primary infection, subsets of memory B and T cells persist upon cytokine survival signals (41, 42). Upon re-encounter with the same antigen, these cells are able to utilize their immunological memory to provide aid to naïve lymphocytes. As a result, secondary and later infections by the same antigen rapidly lead to production of high affinity antibodies and efficient elimination of the pathogen.

Mucosal immune system

The constant exposure to foreign antigens in mucosal tissues, including the intestinal mucosa, sets a challenge for the immune system to initiate appropriate adaptive immune responses to pathogens, while remaining unresponsive towards food antigens and commensal microbes. This is achieved by several functional characteristics that distinguish the mucosal immune system from other lymphoid tissues (reviewed in 43). Once oral tolerance to a certain antigen is acquired, it ideally leads to suppression of all systemic immune responses to this antigen (43). Because of the need to balance between protective immunity and tolerance, the intestinal mucosa is virtually always in a state of physiological inflammation. The final selection between tolerance and elimination is determined by the degree of inflammation in the environment of APCs before antigen presentation to naïve T cells (44).

Central and peripheral tolerance

Cells of lymphoid lineage, including B and T cells, are derived from multipotent hematopoietic stem cells. B lymphocytes mature in fetal liver and after birth in bone marrow, while T lymphocytes originate from fetal liver and bone marrow, but undergo maturation in the thymus.

Both BCRs and TCRs are generated in a highly similar process of VDJ recombination. In B cells, the heavy chain is rearranged first, then the light chain, and if a complete BCR is formed, the immature B cells undergo tolerance to self-antigens (45). Many autoantigens are highly tissue-specific and are not available in the bone marrow or in the circulation. Thus, high numbers of immature self-reactive B cells are released into the circulation, and if activated, they must be eliminated or inactivated. The mechanisms of peripheral tolerance include deletion, anergy, and survival. First, autoreactive B cells that encounter a strongly cross-linking antigen without the presence of infection undergo directly clonal deletion and apoptosis. Second, under exposure to high amounts of antigen, the B cells become unresponsive. Third, immature B cells compete for entry into the B-cell follicles of the spleen, where the follicle provides signals for B-cell survival. In this process, the B-cell-activating factor belonging to the TNF family (BAFF) plays a crucial role.

The precursors of T cells migrate from the bone marrow to the thymus, where their development is directed to one of multiple T-cell lineages (46). In maturing thymocytes, the β-chain of the TCR undergoes rearrangement first, whereafter the α-chain is rearranged, and the thymocyte begins expressing both CD4 and CD8 costimulatory molecules. The double-positive thymocytes that have carried out successful TCR rearrangement enter the phase of positive selection. Cells whose receptors respond to self-peptide–self-HLA complex become selected and mature to either CD4 or CD8 single-

positive cells. These thymocytes then undergo negative selection during which too autoreactive cells are deleted. Negative selection is orchestrated by bone marrow-originated APCs and autoimmune regulator (AIRE) -expressing cells in thymic medulla. Mature T cells resulting from positive and negative selection are HLA-restricted (CD4- or CD8-positive) and tolerant towards autoantigens (47). Since not all autoantigens are expressed in the thymus, a proportion of autoreactive T cells is released into the periphery. This population escaping central tolerance is controlled by mechanisms of peripheral tolerance, including activation-induced cell death, anergy, and tolerance-inducing signals mediated by the lack of costimulatory signaling in the absence of infection.

Autoimmunity

Since autoreactive lymphocytes are always present in the immunological repertoire, mechanisms of peripheral tolerance are necessary to control responses to autoantigens (48). This is ensured by populations of FOXP3 regulatory T cells and induced regulatory T cells that are able to suppress activated weakly autoreactive cells. Lymphocytes are also *per se* prone to apoptosis, causing the immune response to favor self-restricting.

Should the tolerance mechanisms fail, autoreactive lymphocytes may respond to self-antigens. By definition, autoimmunity is mediated by components of the adaptive immune system that cause self-tissue damage such as autoreactive lymphocytes, cytokines, and autoantibodies. Although self-reactive T cells are the main mediators in this process, B cells are also pivotal by providing continuous support to T cells and by enabling epitope spreading over the course of the immune response (49).

Genetic factors, particularly HLA class II molecules, but also several polymorphisms and mutations of other genes have been associated with many autoimmune conditions. However, the dilemma that many genetically susceptible individuals remain unaffected by autoimmune diseases has highlighted the contribution of environmental factors to autoimmunity. Molecular mimicry has been proposed to play a role in autoimmunity. According to this hypothesis, drugs, toxins, or foreign pathogens containing highly similar molecules to self-structures might induce autoimmune responses. Pathogens might promote autoimmunity by causing local inflammation and nonspecific tissue damage, triggering the so-called bystander activation of autoreactive lymphocytes (50).

PATHOGENESIS OF TYPE 1 DIABETES

The prediabetic disease process

Symptomatic type 1 diabetes is preceded by an asymptomatic period, during which autoantibodies against several islet antigens can be detected in the circulation of prediabetic individuals (6). The length of this period has been observed to vary from only a few months to more than two decades (6). The highly variable duration of the prediabetic process suggests that individual genetic, immunological, or environmental factors might modify the pace of the disease progression.

The prediabetic disease process often starts early in life, with the autoantibody seroconversions peaking already during the first or second year of life (51, 52). This indicates that the triggering events of islet autoimmunity and type 1 diabetes occur in infancy or during the fetal period in such cases. The disease may also begin at an older age or in adulthood, but the mechanisms leading to adult-onset

type 1 diabetes are not completely understood and may differ from those causing juvenile diabetes. This thesis focuses on beta-cell autoimmunity in children and adolescents.

A model has been proposed to illustrate the early stages of type 1 diabetes (Figure 1). In this model based on increased genetic risk, stage 1 is defined as the appearance of islet autoantibodies, stage 2 as dysglycemia, and stage 3 as symptomatic type 1 diabetes (53). The asymptomatic phase of type 1 diabetes has been proposed to be given the nomenclature 'Autoimmune Beta Cell Disorder' (ABCD) to emphasize the current view that type 1 diabetes is primarily an immune-mediated disease and only secondarily a metabolic disease (54).

Beta-cell destruction in type 1 diabetes is considered to be a primarily T-cell-mediated process. Islet autoantibodies are considered to merely reflect the disease activity rather than being active players in the pathogenesis. As markers of islet autoimmunity, however, they are suitable for disease prediction. The most commonly used autoantibodies for this purpose are islet cell antibodies (ICA), insulin autoantibodies (IAA), autoantibodies to glutamic acid decarboxylase (GADA), autoantibodies to protein tyrosine phosphatase family member islet antigen-2 (IA-2A), and autoantibodies to zinc transporter 8 (ZnT8A). The characteristics of these autoantibodies are introduced in the following sections.

The appearance of islet autoantibodies does not indicate the extent of beta-cell destruction (55). However, some evidence has supported a more active role for humoral beta-cell autoimmunity in the disease process (56). Also other views on the disease pathogenesis have recently been challenged. Traditionally, it has been believed that 85-95% of beta cells are destroyed at the onset of symptomatic type 1 diabetes and that the rest of the beta cells are permanently lost during a couple of years after diagnosis. Studies on pancreatic islets have, however, indicated that in some patients the remaining proportion of beta cells is significantly higher (57). This has raised questions of whether the severe symptoms of hyperglycemia often observed in overt type 1 diabetes originate from mechanisms other than explicit beta-cell destruction such as cellular stress or dysfunction, inflammation, or other immunological mechanisms. This idea is supported by the considerable heterogeneity in residual beta-cell function among patients with long-duration type 1 diabetes (58, 59).

Considering the pace of the progression from the prediabetic phase to clinical type 1 diabetes, it has been proposed that rapid progression might derive from a congenital defect in the regulation of beta-cell mass, and that individuals with higher beta-cell mass in the early years might present with slower disease progression (57). The human beta-cell mass is established by the age of 5 years, while beta-cell proliferation peaks during the first two years of life, and beta-cell replication in infancy is a substantial determinant of beta-cell mass in later life (60, 61).

According to the so-called "stress test hypothesis", the duration of the prediabetic period might be defined by three aspects: 1) the original quantity of beta-cell mass during early life, 2) the aggressiveness of beta-cell loss during the prediabetic process, determined by the functional capacity of beta cells, the ability of beta cells to survive the immune attack, and the individual genetic, epigenetic, immunological, or environmental factors capable of modifying the pace of the disease progression, and 3) the critical beta-cell mass that is no longer able to maintain sufficient insulin production and drives the disease process over the edge towards the manifestation of symptomatic type 1 diabetes (57).

An overview of the current understanding of the pathogenesis of type 1 diabetes is presented in the following sections.

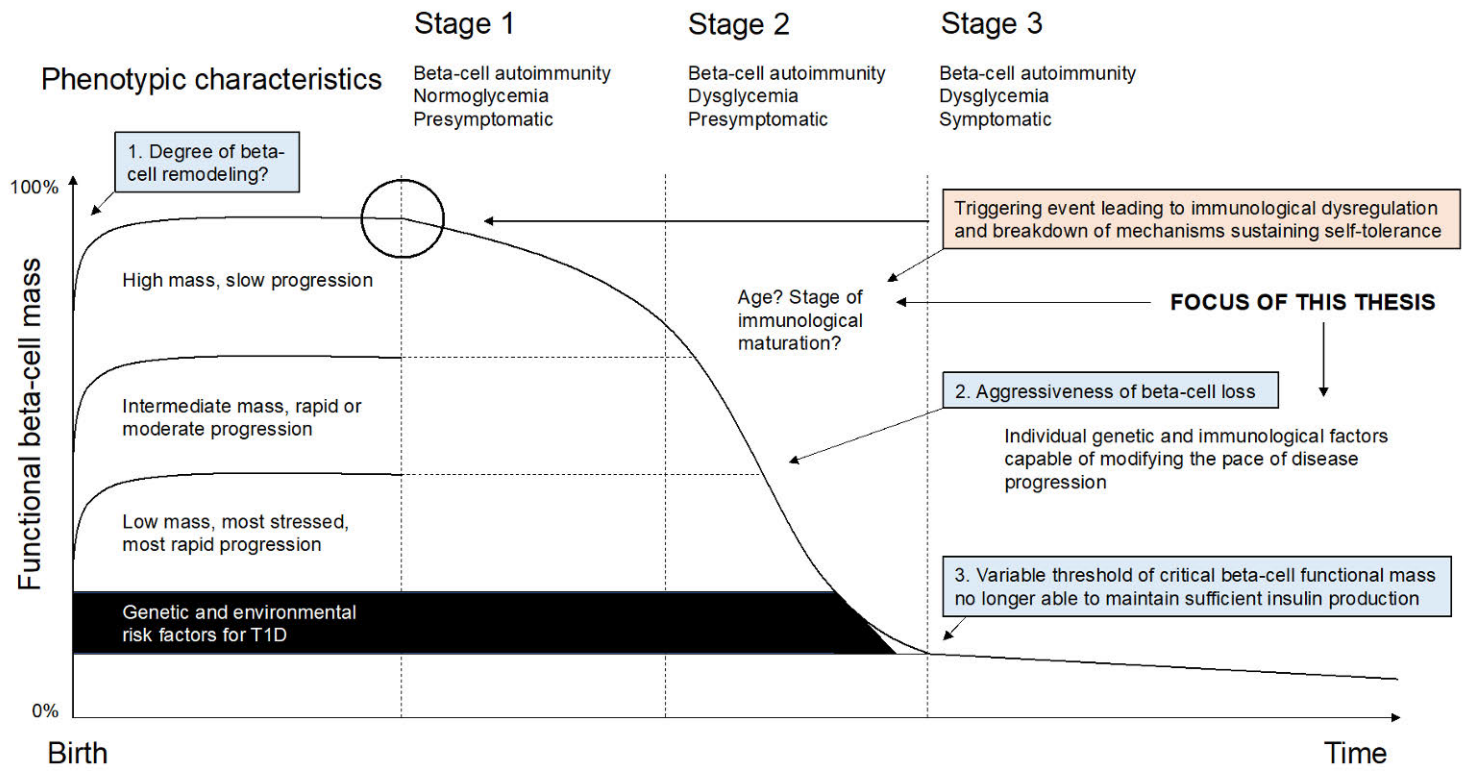

Figure 1. Stages of progression to type 1 diabetes and the factors determining the progression rate in the light of the ”stress test” hypothesis. Immunological dysregulation is an early phenomenon operating already at the beginning of Stage 1. Modified from (53) and (57).

Cell-mediated autoimmunity in type 1 diabetes

Type 1 diabetes is considered to be a T-cell-mediated disease. This is supported by several findings. First, T cells, predominantly CD8 T cells, are found in insulitic lesions of the pancreatic islets (62, 63). Second, hyperexpression of HLA class I molecules in beta cells positive for insulitis suggests direct peptide presentation of beta-cell autoantigens to infiltrating cytotoxic T cells (62–66). Third, several studies have demonstrated the presence of self-reactive T lymphocytes in patients with type 1 diabetes (67–70). Finally, the administration of immunomodulatory therapy affecting the function of T cells has delayed the disease progression in clinical trials (71, 72).

Genetically, the strongest risk for type 1 diabetes is associated with HLA class II genes. Therefore, it might be expected that the autoimmune response is initially driven by a peptide antigen, and that CD4 T cells are involved in the process. Rather than through direct contact with the beta cells, CD4 T cells might cause beta-cell damage by cytokine secretion, by supporting autoantibody production, and by promoting the function of cytotoxic T cells (73). Yet, some beta cells of subjects with type 1 diabetes express HLA class II molecules, suggesting a direct interaction between beta cells and CD4 T cells in the affected islets (74).

Several alterations in CD4 T-cell subsets have been observed in patients with type 1 diabetes and prior to clinical disease (75–77). The frequency of follicular T helper cells is increased in children with multiple islet autoantibodies and impaired glucose tolerance (IGT) (77). Similarly, circulating CXCR5+PD-1^{hi} peripheral T helper cells have been associated with progression to clinical disease (78). In the Type 1 Diabetes TrialNet study, distinct immune phenotypes of T and B cells were seen depending on the stage of disease progression (79). Also an attenuated cytokine response to IL-2, but an enhanced response to IL-6 in CD4 T cells might play a role (79–82). Changes in regulatory T-cell subsets might be related to more advanced stages of the disease process (83).

Recently, lymphocytes that expressed both BCRs and TCRs and the key lineage markers of T and B cells were discovered in the peripheral blood of patients with type 1 diabetes (84). Furthermore, some evidence has emerged for the role of neutrophils in the process. Reduced number of neutrophils in peripheral blood has been associated with worsening of the beta-cell function (85, 86).

Insulitis, pancreatic size, and beta-cell properties

Insulitis is a central phenomenon in the pathogenesis of type 1 diabetes. However, the proportion of simultaneously affected pancreatic islets at a given time is modest (87). Insulitis is more common in young children than adolescents, shows an inverse correlation with disease duration, and affects mostly pancreatic islets containing insulin (87–89). The criteria for insulitis have been defined as the infiltration of lymphocytes in the pancreatic islets of Langerhans with a count of at least 15 CD45 cells within an islet and in at least three islets simultaneously (90). The insulitic lesion may contain both T and B lymphocytes, the cytotoxic CD8 T cells being the prevailing subset (89). Autoreactivity of the infiltrating CD8 T cells has been demonstrated (67).

A prominent feature of human type 1 diabetes is hyperexpression of HLA class I molecules in beta cells positive for insulitis (65, 66). Both the presence of insulitis and the hyperexpression of HLA class I molecules might indicate chronic islet inflammation (65). Signs of persistent enteroviral infection have, in fact, been detected in the pancreatic islets of living patients with type 1 diabetes (91). This suggests that CD8 T cells present in the insulitic lesion might recognize viral epitopes

derived from the infected beta cells of HLA class I molecules (67, 91). Furthermore, IFN-γ, a central cytokine in immune response against viral infections, has been shown to mediate the hallmarks of insulitis: HLA class I hyperexpression, beta-cell apoptosis, and endoplasmic reticulum (ER) stress in pancreatic islets (92). Heterogeneity in the features of insulitis exists since there are at least two distinct phenotypes of insulitis. They are characterized by either high or low frequency of CD20-positive B cells in the insulitic lesion and are associated with a considerably different age at onset of clinical diabetes (64, 89, 93).

Considering the severe symptoms and deteriorated insulin secretion seen at diagnosis of type 1 diabetes, insulitis findings present at diagnosis are relatively minor (87). Also the residual beta-cell mass and the proportion of surviving beta cells at diagnosis are higher than previously expected, suggesting that the severe hyperglycemia observed in many cases at diagnosis of diabetes might derive from mechanisms outside of direct beta-cell damage such as inflammation, cellular stress, or beta-cell dysfunction (94). Beta cells persist long after the onset of symptomatic disease and may be able to recover after damage or dysfunction (95). In several studies, type 1 diabetes cases have been described in which C-peptide production has been measurable after long disease duration, proposing an incomplete loss of beta cells (58, 59, 94).

How the surviving beta cells escape autoimmune destruction and whether their dysfunction is reversible are not yet fully understood. Most of the residual insulin secretion in type 1 diabetes might, in fact, derive from dedifferentiated beta cells or non-beta endocrine cells rather than from preserved beta cells (96–99). In any case, restoring the residual insulin production comprises a fascinating approach to prevention and treatment of type 1 diabetes. Apart from the honeymoon period shortly after diagnosis, there is no evidence of functional recovery of beta cells in established type 1 diabetes (94). Some remission of the beta cells might be possible close to the diagnosis (95).

Pancreatic size is diminished in type 1 diabetes (100). Interestingly, also autoantibody-negative first-degree relatives (FDRs) of patients with type 1 diabetes exhibit smaller pancreatic size than controls with no affected relatives (101). The appearance of islet autoantibodies does not imply the loss of beta-cell mass (55).

To summarize, insulitis as part of the pathogenesis of type 1 diabetes seems to be a chronic, and possibly heterogeneous condition that takes place in only a small fraction of pancreatic islets at a given time and continues years after onset of overt disease. However, as beta cells in type 1 diabetes seem to be preserved to a higher extent than previously thought, not all affected beta cells are necessarily under attack of the immune system. Beta-cell senescence may also play a role in the disease pathogenesis (102).

Humoral beta-cell autoimmunity

Type 1 diabetes-associated autoantibodies

Islet cell autoantibodies, ICA

Islet cell antibodies (ICA) were the first described diabetes-associated autoantibodies (16, 103). They are analyzed by using a standardized immunofluorescence staining method on sections of frozen human pancreatic tissue (16). The ICA assay measures immunoglobulin (mostly IgG) binding on various structures of the pancreatic islets. Since the assay is labor-intensive and challenging to standardize and necessitates access to high-quality pancreatic tissue from organ donors, it has become tempting to replace ICA with biochemical autoantibodies for screening purposes. ICA are not beta cell-specific, but some of the reactivity is derived from other islet autoantibodies and also reactivity to unidentified antigens contributes to ICA reactivity (104, 105). The predictive characteristics of ICA have been extensively studied. Low ICA titers are widely detected in the general population and relatives of patients with type 1 diabetes with an increasing frequency in older subjects, suggesting that positivity for ICA in the absence of other autoantibodies represents non-progressive beta-cell autoimmunity (106, 107). In contrast, high ICA titers are associated with increased risk for type 1 diabetes and are seen mostly in association with positivity for multiple biochemical autoantibodies (8, 108, 109). Positivity for ICA has been associated with female gender, the *HLA-DR4-DQ8* haplotype, and young age at disease onset (110).

Autoantibodies against insulin, IAA

Autoantibodies to insulin (IAA) were first described in 1983 in patients with recent-onset type 1 diabetes (111). Among the islet autoantibodies, IAA appear often as the first or among the first autoantibodies (52, 112–114). In the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study, seroconversions to IAA positivity peaked during the second year of life, while in The Environmental Determinants of Diabetes in the Young (TEDDY) study a peak was seen already during the first year (51, 52). Despite the early appearance during the disease process, IAA are rather unstable (112, 114, 115). Positivity for IAA has been associated with the *HLA-DR4-DQ8* haplotype mediating increased risk for type 1 diabetes (116–118). Also several non-HLA gene polymorphisms have been associated with IAA positivity, including the insulin-encoding *INS* gene variable number tandem repeat (VNTR) polymorphism and single-nucleotide polymorphisms (SNPs) in the *INS* (rs689) and lymphoid tyrosine phosphatase-encoding *PTPN22* genes (rs2476601) predisposing to type 1 diabetes (110, 119–121). In contrast, the predisposing SNP in the IKAROS family zinc finger 4 (*IKZF4*) gene (rs1701704) has been inversely associated with IAA positivity (120). In young children, IAA have high sensitivity for predicting clinical type 1 diabetes (7, 112). High initial IAA titers increase the risk of progression to clinical disease (8, 51, 108). Recently, autoantibodies against a posttranslationally modified neoautoantigen, oxidized insulin, have been reported to improve the prediction of type 1 diabetes compared with autoantibodies against native human insulin (122).

Autoantibodies against glutamic acid decarboxylase, GADA

The enzyme glutamic acid decarboxylase (GAD) catalyzes the synthesis of the neuroinhibitory transmitter gamma-aminobutyric acid (GABA) from glutamate and is a major autoantigen in type 1 diabetes. Within the beta cell, the 65 kD isoform of GAD is localized in synaptic-like microvesicles. Autoantibodies against GAD (GADA) were first recognized as autoantibodies to a 64 kD cytosolic protein that was later identified as the 65 kD GAD protein (123, 124). There are two isoforms of the GAD protein with molecular weights of 65 and 67 kD, but only the former is expressed in the pancreas (125). Positivity for GADA has been associated with female gender and the *HLA-DR3-DQ2* haplotype predisposing to type 1 diabetes (110, 117, 126, 127). Polymorphisms in the *IKZF4-ERBB3* region (rs1701704, rs2292239) have been reported to affect the rate of beta-cell damage specifically among children with GADA as the primary autoantibody, and the predisposing *BACH2* gene SNP (rs3757247) increases the risk of developing positivity for GADA only (128, 129). GADA are not specific for pancreatic islets and have been indicated to be associated with autoimmunity in general (118). Unlike high IAA or IA-2A titers, GADA titers do not increase the risk of type 1 diabetes among children with persistent islet autoantibodies (130). Also among siblings of affected children, GADA titers are not related to increased risk for type 1 diabetes, although the risk is increased in the presence of high levels of ICA, IAA, and IA-2A (131). At the diagnosis of type 1 diabetes, 65–68% of Finnish pediatric patients aged 0.01–16.0 years test positive for GADA (132, 133). After the diagnosis, GADA levels remain elevated longer than those of other islet autoantibodies (134).

Autoantibodies against N-terminally truncated glutamic acid decarboxylase, truncated GADA

A proportion of individuals with autoantibodies against the full-length GAD, comprising amino acid residues 1–585, do not progress to clinical type 1 diabetes. Therefore, it has become necessary to find more specific GADA assays. Autoantibodies against N-terminally truncated GAD, covering amino acids 96–585, have similar sensitivity, but are associated with a higher risk of type 1 diabetes than the full-length GADA among GADA-positive FDRs of patients with type 1 diabetes (135). This suggests that the truncated GADA might be beneficial in identifying participants for future intervention trials aimed at preventing type 1 diabetes. Among patients with adult-onset diabetes, truncated GADA positivity is associated with a clinical phenotype of autoimmune diabetes and predicts the need for insulin treatment (136). The initial GAD response is directed mostly to the M- and C-terminal epitopes of the GAD protein in progressors to type 1 diabetes, whereas immune responses to the N-terminal epitope do not increase the disease risk (137, 138). Epitope spreading during the GAD-specific autoimmune response has been demonstrated to occur from the middle region to both ends of the protein (139, 140).

Autoantibodies against islet antigen-2, IA-2A

Islet antigen-2 (IA-2) autoantibodies represent antibody reactivity against the intracellular part of the transmembrane protein islet cell antigen 512, which is a member of the family of protein tyrosine phosphatases. Islet antigen-2 was first identified as a protein fragment of molecular weight 40 kD, which among two other 50 kD and a 37 kD fragments resulted from proteolytic trypsin-mediated digestion of GAD (64 kD islet cell protein) (141–143). The 37 kD fragment was observed to be

derived from a closely related transmembrane protein tyrosine phosphatase IA-2β (phogrin), which is also a major autoantigen in beta-cell autoimmunity and shares homology with the cytoplasmic domain of IA-2, but IA-2 is the primary autoantigen associated with type 1 diabetes (144, 145).

During islet autoimmunity IA-2A are often the last or among the last autoantibodies, but rarely the first autoantibody to appear (51, 112, 146). They are the most specific autoantibodies for predicting type 1 diabetes and are associated with a high risk of progression to clinical disease (112, 147, 148). The disease risk associated with IA-2A is higher than that of GADA or IAA (7, 149). Early appearance of IA-2A in the prediabetic process is also associated with accelerated disease progression (150, 151). The appearance of IA-2A positivity has been associated with the *DQB1*03:02/*x genotype (x=other than *DQB1*02* allele) (126, 132), and high IA-2A levels with the *HLA-DR4* haplotype (118, 152). High IA-2A levels increase the disease risk among persistently autoantibody-positive children (130). In young multipositive children, high IA-2A levels predict progression to clinical disease (153). In Finnish children between 0.01 and 16.0 years of age, the prevalence of IA-2A at diagnosis of type 1 diabetes is 75–79% (132, 133).

Autoantibodies against zinc transporter 8, ZnT8A

The cation efflux transporter zinc transporter 8 (ZnT8) is the most recently discovered autoantigen in patients with type 1 diabetes (154). The ZnT8 is primarily located in the islets of Langerhans, with the highest expression in beta cells, where it serves as the most abundant zinc transporter, participates in the regulation of zinc accumulation into insulin secretory granules, and is involved in insulin secretion (155, 156). Hexamers containing six insulin molecules together with two Zn^{2+} ions are stored in secretory vesicles in the beta cell (156). The ZnT8 molecule is a 369 amino acid polyepitopic transmembrane protein that contains cytoplasmic C- and N-terminal domains (154).

The ZnT8 protein is encoded by the *SLC30A8* gene, which has been shown to affect insulin production (157). A SNP in the *SLC30A8* gene that results in a modification in the protein amino acid sequence at position 325 has been associated with both type 2 (158) and type 1 diabetes (159). The ZnT8 autoantigen has three variants at amino acid position 325 determined by the *SLC30A8* polymorphism: ZnT8-R (arginine), ZnT8-W (tryptophan), and ZnT8-Q (glutamine) (160). Accordingly, ZnT8A against the C-terminal are amino acid-specific, and this epitope specificity is defined by the *SLC30A8* genotype (161, 162).

At diagnosis of type 1 diabetes, ZnT8A positivity has been inversely associated with the *HLA-DR3-DQ2* haplotype and the *DR3-DQ2/DR4-DQ8* genotype predisposing to type 1 diabetes (163–165). However, the negative association of the *DQ2* haplotype with ZnT8A is restricted to ZnT8WA (tryptophan) and ZnT8QA (glutamine), but not ZnT8RA (arginine) (165). Both *DQ8* (without *DQ2*) and *DQ6.4* (without *DQ8* or *DQ2*) haplotypes have been associated with ZnT8A regardless of the amino acid variant (165). The differences in the HLA associations might be related to distinct binding of DQ molecules to ZnT8 epitopes (166). The type 1 diabetes risk-associated HLA-DR3 and -DR401 molecules bind to peptides derived from ZnT8 (167). An *AGER* gene polymorphism might also affect the frequency of ZnT8A positivity (164).

In type 1 diabetes, ZnT8 is a target antigen of IFN-γ-producing autoreactive T cells (167). A broad repertoire of ZnT8-derived peptides is recognized by CD8 T cells, which might suggest that epitope spreading along the ZnT8 protein occurs during the autoimmune response (168–170). Functional differences in ZnT8-specific T cells have also been found between patients with type 1

diabetes and non-diabetic individuals, and these autoreactive cells are abundant in the pancreas of subjects affected by type 1 diabetes (171).

In new-onset type 1 diabetes, ZnT8A are present in approximately 60–70% of patients (159). Accordingly, in Finnish children with type 1 diabetes at the age of 0.3–15.0 years the prevalence of ZnT8A at diagnosis is 63% (164). At diagnosis, ZnT8A positivity has been associated with reduced beta-cell function and higher rate of ketoacidosis, and after the diagnosis with higher insulin requirement (163). However, contradictory observations on the risk of ketoacidosis have also been reported in a study cohort comprising more recently born children (164). The combined measurement of ZnT8A with other biochemical autoantibodies improves the detection of islet autoimmunity (154, 159, 172), although in the Finnish pediatric population tested at diagnosis the improvement was minimal (164). Altogether, ZnT8A have been considered to appear at an older age and in a later phase of the disease process than other islet autoantibodies, and are often detected together with ICA and IA-2A, but less frequently with simultaneous positivity for GADA or IAA at diagnosis (154, 159, 163). After diagnosis, ZnT8A disappear more rapidly than GADA or IA-2A (154). ZnT8-targeted T-cell responses are also attenuated shortly after diagnosis (170).

Neoepitopes as targets of beta-cell autoimmunity

An increasing body of evidence implies that neoepitopes formed by post-translational modification of self-proteins might be involved in the pathogenesis of type 1 diabetes as a target of autoreactive T-cell responses (173). In the future, such neoantigens might prove useful in the disease prediction.

Spreading of the autoimmune response

Epitope spreading is a frequent phenomenon during the prediabetic disease process and occurs already early in the process both within an autoantigen and from one autoantigen to another (139, 144, 174). Whereas positivity for a single autoantibody does not confer any notable risk for type 1 diabetes, the presence of two or more biochemically defined autoantibodies is associated with a highly increased risk (149). Epitope spreading to positivity for multiple autoantibodies develops usually within the first year after the initial seroconversion and rarely thereafter (146, 175).

Epitope-specific humoral responses

The disease risk associated with epitope-specific autoantibodies to the major autoantigens varies considerably. The middle and C-terminal epitopes of GAD are associated with increased risk for type 1 diabetes, whereas the N-terminal GAD-epitope confers no risk for multipositivity or type 1 diabetes (176, 177). More specifically, the first 142 amino acids of GAD are not recognized by risk-associated autoantibodies (178). The initial GADA response is M-epitope-specific and spreads rapidly to the C-terminal epitope, but rarely to the N-epitope (137, 139, 179). High GADA titers are associated with broader epitope reactivity (140). Autoantibody responses to the full-length GAD compared with the N-terminally truncated GAD are more common among healthy individuals than people with type 1 diabetes, which might result from reduced presentation of two or more conformational epitopes by the N-terminally truncated GAD (180). Most IA-2A identify the cytoplasmic part of the IA-2 protein, whereas no reactivity has been detected towards the transmembrane or extracellular parts (181, 182).

In young children, IA-2A towards the juxtamembrane epitopes tend to appear first, before autoantibodies against the protein tyrosine phosphatase epitopes, and this type of response is characteristic of children who later progress to type 1 diabetes (183). The mature autoimmune response against ZnT8 occurs mostly against the C-terminal epitopes of the antigen (154). Patients with type 1 diabetes have single amino acid-specific autoantibodies against the ZnT8-R and ZnT8-W variants, the latter of which might have higher affinity for their specific autoantigen than the former (162). However, ZnT8A comprise also a variety of responses to epitopes unaffected by the amino acid in position 325 (161).

Isotype-specific humoral responses

During the prediabetic period also switching of the immunoglobulin class within the same autoantigen takes place. At the diagnosis of type 1 diabetes, IAA are primarily of the IgG1 subclass and to a lesser extent IgG4 and IgG2 subclasses (184). In young children, the IAA-related risk for type 1 diabetes is mostly associated with the IgG1 subclass, followed by IgG4, IgG3, and IgG2 (185). Strong IgG1 and IgG3 subclass IAA responses increase the risk of type 1 diabetes, while weak or lacking IgG3 responses might mediate some degree of protection against the clinical disease (186). Similarly, GADA are mostly of the IgG1 subclass in both patients with type 1 diabetes and individuals at high disease risk (148, 187). Type 1 diabetes has been associated with IgG1, IgG2, and IgG3 subclass GADA, while a broad early response to IgG3 and IgG4 might mediate a protective effect against overt disease (137). Also IgG2 and IgG4 subclass GADA responses tend to be more common among non-progressors than progressors, although no significant differences in the distributions have been reported (138). In the case of IA-2A, the humoral immune response comprises mainly IgG1 subclass in both type 1 diabetes patients and prediabetic individuals (187). Non-diabetic children positive for IA-2A present more often with IgE class IA-2A and have higher titers of IgE class IA-2A than those who develop clinical diabetes (183). Among children with recent-onset diabetes, those who test positive for IA-2A only out of three biochemical autoantibodies (IAA, GADA, IA-2A) demonstrate a broader IA-2 isotype-specific response and a stronger association with the high-risk *HLA-(DR4)-DQB1*03:02* haplotype than those with all three biochemical autoantibodies (133). In conclusion, most diabetes-associated autoantibodies are composed mainly of IgG class, indicating that maturation of the humoral immune response has occurred.

Autoantibody affinity

In general, high-affinity antibodies are a sign of a mature humoral immune response. After encountering their specific antigen, B cells migrate to lymph nodes, where they undergo somatic hypermutation that allows them to produce high-affinity antibodies over the course of several months from the initial encounter with the antigen. In type 1 diabetes, high-affinity IAA increase the risk of clinical diabetes (188, 189). However, the affinity of IAA does not seem to affect the progression rate from seroconversion to clinical disease (190). Similarly, high affinity of GADA confers higher susceptibility to type 1 diabetes than low-affinity GADA (176). This may be related to the spreading of the autoimmune response because children positive for only GADA show lower affinity of GADA than those with multiple autoantibodies (176). High-affinity GADA is more common among children carrying the *HLA-DR3* haplotype (176).

Transient autoantibodies

Non-persistent positivity for islet autoantibodies has been considered to be a relatively uncommon phenomenon and has not shown any correlation with the identified genetic risk factors (115, 191, 192). However, in children with increased HLA susceptibility to type 1 diabetes, transient positivity for IAA occurs frequently during the first two years of life (112). Persistent IAA has been associated with a higher risk of type 1 diabetes than fluctuating IAA (8, 130, 193), and there has been some evidence that transient autoantibodies might reflect the pace of the disease progression. In the BABYDIAB study, a lower proportion of primarily IAA-positive multipositive children who became IAA-negative had progressed to clinical diabetes in 10 years than children who remained IAA-positive (194). In contrast, transient GADA positivity in the presence of persistent IAA and IA-2A positivity has been associated with more rapid disease progression than stable GADA (195). Reversion of multiple autoantibody positivity occurs rarely, but is associated with a reduced risk of progression to type 1 diabetes (196).

Are autoantibodies only bystanders in the disease process?

Finally, it has been under debate whether the humoral autoimmunity in the pathogenesis of type 1 diabetes is only secondary to T-cell-mediated autoimmunity. Islet antigen-specific B cells are central in the development of islet autoimmunity and are likely to provide support to T cells in the process of beta-cell destruction. Accordingly, the involvement of autoreactive B cells indicates the failure of silencing mechanisms of immunological tolerance. The loss of anergic B-cell populations has, in fact, been associated with islet autoimmunity and progression to type 1 diabetes, proposing that events disrupting the B-cell anergy might cause deviation towards islet autoimmunity among individuals at genetic risk (197). Moreover, several disturbances of B-cell responses have been associated with islet autoimmunity and clinical diabetes in the TrialNet study, including a decrease in the frequency of anergic B cells in autoantibody-positive individuals, and decreased B-cell responses towards the progression to clinical disease (79). Most importantly, selective depletion of B cells in the rituximab trial resulted in delayed disease progression in newly diagnosed patients with type 1 diabetes (56).

ETIOLOGY OF TYPE 1 DIABETES

Genetics

Human leukocyte antigen (HLA)

HLA structure and function

HLA molecules are glycoproteins located on the cell surface that present peptide antigens derived from microbial pathogens and host tissues for recognition to T lymphocytes. HLA class I molecules are expressed in all nucleated cells, but expression of HLA class II molecules is limited to APCs.

Multiple genes encode HLA molecules, leading to a highly individualized repertoire of HLA molecules with different antigen-binding properties. The main HLA-encoding genes are located on the short arm of chromosome 6. In this region, there are three pairs of genes encoding HLA class II α- and β-chains, the *HLA-DR*, *-DQ*, and *-DP*, and three genes encoding class I α-chain, the *HLA-A*, *-B*, and *-C*. The HLA-encoding genes are highly polymorphic with multiple variants of each gene found within a population, except for the non-polymorphic β-chain of HLA class I molecules, β2-microglobulin, which is encoded outside the HLA region on chromosome 15.

Four types of HLA class II molecules can be formed from the three pairs of genes. The different HLA alleles, the *DR* and *DQ* loci in particular, are in strong linkage disequilibrium (LD) with each other. Haplotypes formed by the combinations of these alleles have been associated with several autoimmune conditions.

The HLA alleles differ in amino acid sequence, especially on the peptide-binding site and the regions exposed to cell surface that bind to TCRs (198). Therefore, each HLA molecule exhibits different binding specificity for peptide antigens and T-cell recognition is restricted to a specific HLA variant. This means that T cells are only able to recognize their TCR-specific antigen when it is bound to a particular HLA molecule.

The HLA gene polymorphisms determine the range of peptides bound by HLA molecules and the binding of the HLA-peptide complex to a certain TCR. This extensive variability enables a broad range of antigen recognition against invading pathogens and creates highly personalized immune responses — each individual responds differently to a given antigen.

HLA class II effects on type 1 diabetes

The inherited risk for type 1 diabetes is mostly determined by the HLA class II genes, mediating approximately 40–50% of the genetic risk for type 1 diabetes (199). Polymorphisms in *HLA-DRB1*, *DQA1*, and *DQB1* genes confer susceptibility to type 1 diabetes. Most of the disease risk mediated by these genes in populations of European origin is determined by three amino acid positions (200). A strong LD connects these genes since the alleles of different loci are inherited together as certain haplotypes more often than expected given their frequencies in the population. The combination of two inherited haplotypes, one from the mother and the other from the father, determines an individual's HLA-mediated risk for type 1 diabetes.

The haplotypes formed by combinations of the HLA class II genes can be classified according to the risk they confer for type 1 diabetes, ranging from high risk to strong protection (201). The disease risk mediated by some common haplotypes is shown in Table 1. The highest risk is mediated through a heterozygous genotype comprising the two major risk-associated serologically defined haplotypes, the *DR3-DQ2* (*DRB1*03:01-DQA1*05(:01)-DQB1*02*) on one chromosome and the *DR4-DQ8* [*DRB*04:01/02/04/05/08-DQA1*03(:01)-DQB1*03:02(/04)*] on the other (202). In Finland, the *DR4-DQ8* is the most common haplotype conferring increased risk for type 1 diabetes, followed by the *DR3-DQ2* (203). Due to the enhanced contribution of environmental factors to the pathogenesis of type 1 diabetes, the HLA associations behind the disease process are changing and an increasing proportion of individuals with "lower" HLA-mediated disease risk are progressing to clinical type 1 diabetes (204).

Table 1. Classification of genetic risk for type 1 diabetes mediated by some common HLA class II haplotypes (A) and genotype risk categories for type 1 diabetes defined by the combination of HLA class II haplotypes (B). Modified from (201).

A

Haplotype	Odds Ratio	Risk
DRB1*04:01-DQA1*03-DQB1*03:02	10.1	S
DRB1*04:05-DQA1*03-DQB1*03:02	3.0	S
DRB1*04:04-DQA1*03-DQB1*03:02	2.8	s
(DR3)-DQA1*05-DQB1*02	2.8	s
DRB1*04:02-DQA1*03-DQB1*03:02	1.8	S
(DR13)-DQB1*06:04	1.1	N
(DR9)-DQA1*03-DQB1*03:03	1.0	N
(DR8)-DQB1*04	1.0	N
(DR16)-DQB1*05:02	0.8	N
(DR7)-DQA1*02:01	0.6	N
(DR1/10)-DQB1*05:01	0.6	N
(DR4)-DQA1*03-DQB1*03:01	0.5	N
DRB1*04:03-DQA1*03-DQB1*03:02	0.4	p
(DR13)-DQB1*06:09	0.4	N
(DR13)-DQB1*06:03	0.2	p
(DR11/12/13)-DQA1*05-DQB1*03:01	0.2	p
(DR7)-DQA1*02:01-DQB1*03:03	0.08	P
(DR15)-DQB1*06:01	0.07	P
(DR15)-DQB1*06:02	0.03	P
(DR14)-DQB1*05:03	0.03	P

B

Genotype risk category	Haplotype risk code combination
High risk	S/s, s/s (if *DR3-DQ2*/*DR4-DQ8* genotype)
Moderately increased risk	S/s, s/s (not *DR3-DQ2*/*DR4-DQ8*), S/S, S/N
Slightly increased risk	s/N, S/p
Neutral	N/N, S/P, s/P, s/p
Slightly decreased risk	p/N
Strongly decreased risk	P/N, p/p, P/p, P/P

S=strong susceptibility, s=weak susceptibility, N=neutral, p=weak protection, P=strong protection

HLA class I effects on type 1 diabetes

Apart from HLA class II *DR/DQ* region determinants, also HLA class I genes and other loci outside the HLA region have been associated with increased risk for type 1 diabetes (205, 206). In the Finnish population, *HLA-B*39:06* allele increases the risk for type 1 diabetes among individuals with the (*DR8*)-*DQB1*04* haplotype (207). The HLA class I alleles affect also the pace of disease progression, as described in the following sections.

Genetic factors outside the HLA region

Non-HLA gene associations with type 1 diabetes

At present, more than 60 non-HLA gene loci have been confirmed to affect the risk of type 1 diabetes, and these loci together with HLA genes have been estimated to be responsible for over 80% of the heritability of the disease (208, 209). Large-sample genome-wide association studies (GWAS) have contributed to the discovery of multiple new non-HLA risk loci for type 1 diabetes (210–220). However, since the GWAS include only relatively common SNPs in the population, excluding rare disease variants, they are at best able to identify only a limited fraction of the genetic variants that mediate the risk for type 1 diabetes. Therefore, a 200 000 SNP ImmunoChip array was developed that provides a powerful tool for the detection of rare gene variants associated with the risk of immune-mediated diseases. Several findings from the ImmunoChip analyses on type 1 diabetes have been published (221–230). The unexplained proportion of the heritability of type 1 diabetes has been suggested to result from the effect of unidentified non-HLA genes, structural modification of gene material, or epistatic interactions between genes. Epigenetic factors may also play a role.

Insulin gene (INS)

After the HLA region, the insulin gene (*INS*) locus on chromosome 11p15 comprises the second most important genetic risk association with type 1 diabetes. Originally, the susceptibility locus for type 1 diabetes within the *INS* gene was pinpointed to a VNTR region, which is characterized by three classes of alleles according to the number of repeats (231). The highest risk for type 1 diabetes is associated with the shortest repeats (class I alleles). Later on, two *INS* SNPs, –23HphI (rs689) and +1140A/C (rs3842753), have been consistently associated with type 1 diabetes in several studies (232). In populations of Caucasian origin, the rs689 SNP is found in complete LD with the VNTR region such that the predisposing SNP allele A is linked to short class I VNTR alleles, while the non-predisposing SNP allele T is linked to long class III VNTR alleles (233). The former (risk-conferring) genotype has been associated with higher insulin expression in the human pancreas, but lower expression in the thymus, whereas the latter (non-predisposing) genotype shows the opposite association. Accordingly, the *INS* polymorphisms might affect the development of central tolerance to insulin by regulating the abundance of insulin miRNA and protein expression in the thymus (234, 235). Also, another type 1 diabetes-associated SNP, -2221MspI (rs3842729), is situated within the *INS* gene. In the case of this SNP, the predisposing allele C is in LD with the VTNR class I and subclass IIIB alleles, whereas the non-predisposing allele T is associated with VNTR subclass IIIA (236).

The predisposing *INS* SNP rs689 has been associated with the appearance of IAA as the primary autoantibody, but not with the pace of progression to clinical disease after seroconversion (117, 128). The association with the appearance of humoral islet autoimmunity is restricted to individuals with initial IAA positivity and has not been seen for those with GADA as the primary autoantibody (128).

Protein tyrosine phosphatase, non-receptor type 22 (PTPN22)

The *PTPN22* gene is located on chromosome 1p13 and encodes the lymphoid tyrosine phosphatase (LYP), which functions to prevent spontaneous T-cell activation and possesses the ability to inhibit the function of TCRs. The association between *PTPN22* and type 1 diabetes is mediated through a gain-of-function SNP (rs2476601) at amino acid position 620 that causes a change in the amino acid sequence from arginine to tryptophan (237). However, the exact mechanism behind the increased susceptibility for type 1 diabetes and other autoimmune diseases remains open. The altered function of immune cell signaling might affect thymic negative selection or impair the function of regulatory T cells, which would allow autoreactive T cells to act unguarded. According to recent evidence, LYP together with another type 1 diabetes-linked protein tyrosine phosphatase is induced following the activation of naïve T cells, and by controlling the JAK-STAT pathway might be involved in the determination of how activated or memory T cells interpret and respond to cytokine signals (238). In addition, the *PTPN22* risk allele A has been linked to an increased frequency of circulating regulatory T cells (239). The *PTPN22* SNP predisposing to type 1 diabetes has shown an association with both the appearance of islet autoantibodies and progression from seroconversion to clinical disease (240), but no associations with specific autoantibody signatures have been observed.

Familial type 1 diabetes

Although most cases of pediatric type 1 diabetes are sporadic, having an FDR affected by type 1 diabetes increases the risk of type 1 diabetes 8–15-fold and an affected second-degree relative 2-fold compared with the general population (241, 242). The risk of type 1 diabetes is higher in children with a father affected by type 1 diabetes than in children with an affected mother (241).

Epigenetic factors

In brief, the epigenome modulates the accessibility of DNA for transcription factors that control the level of gene expression in response to environmental interactions. Epigenetic regulation is essential for the integration of endogenous and exogenous signals to ensure appropriate gene expression. The epigenome comprises modifications of gene expression that do not involve changes in the DNA sequence, but can be inherited. These mechanisms include e.g. DNA methylation, post-translational histone modifications, gene silencing, regulation of gene expression by non-coding RNAs, and X chromosome inactivation (243). Type 1 diabetes-linked DNA methylation variable positions have been reported among monozygotic twins discordant for type 1 diabetes, including hypomethylation of the risk-associated *HLA-DQB1* gene and the *GAD2* gene, which encodes the autoantigen GAD (244). Variation of DNA methylation in the promoter region of the *INS* gene has also been reported (245). Some changes have been found in the patterns of histone modification and RNA interference between patients with type 1 diabetes and non-diabetic controls (245). Longitudinal differences in

DNA methylation profiles were observed in cases with type 1 diabetes prior to diagnosis in the Diabetes Autoimmunity Study in the Young (DAISY) (246).

Environmental risk factors

Microbial etiology

Hygiene and biodiversity hypotheses

Originally, the hygiene hypothesis suggested that reduced microbial contacts in infancy might increase the susceptibility to allergic or immune-mediated diseases. Several infections have been inversely associated with the risk of allergic conditions (247). Early microbial exposure might be pivotal for the education of the immune system in infancy (248). However, the hygiene hypothesis might not be entirely true since some studies have provided contradictory evidence, including the association of early life infections with islet autoimmunity (249–252). Instead, the role of exposure to commensal microbes in the environment has become of interest. According to the biodiversity hypothesis, the human microbiota might be unfavorably altered in the urban living environment, and this might increase the risk of allergic and immune-mediated diseases (253). In fact, having an indoor dog as a pet in infancy has been shown to decrease the risk of beta-cell autoimmunity (248).

Viral infections

Seasonal pathogens, especially viruses, have been proposed to play a role in the pathogenesis of type 1 diabetes. The strongest evidence for an association with type 1 diabetes exists for enteroviruses, in particular group B coxsackieviruses, but some studies have supported a role for many other viruses, including rotaviruses (254, 255). Moreover, early cytomegalovirus infection might delay the onset of clinical diabetes (256). During the present coronavirus disease 2019 (COVID-19) pandemic concerns have been raised about the comorbidity between the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and type 1 diabetes, especially as the infection might induce direct beta-cell damage or dysfunction (257).

Currently, the primary hypothesis is that enteroviruses cause an acute infection of the pancreatic beta cells. Failure to eradicate the virus might lead to persistent chronic low-grade infection, resulting in beta-cell destruction and type 1 diabetes (258). This is supported by several findings. Increased frequencies of enteroviral RNA have been detected in both stools and blood before the onset of islet autoimmunity (259, 260). Coxsackie B viruses show tropism to human beta cells, and enteroviruses are found in the pancreatic islets of most patients with type 1 diabetes (91, 261). The main receptor for group B coxsackieviruses is expressed in the pancreatic islets (261, 262). In epidemiological studies, the association between enteroviruses and type 1 diabetes has been confirmed in a meta-analysis (263). A systematic analysis of the specific enterovirus types revealed that the highest risk was associated with coxsackievirus B1 infections (264). Also, prospective intestinal virome analyses in HLA-predisposed children have suggested that a chronic enteroviral infection is associated with the development of islet autoimmunity (265). Interestingly, the coxsackievirus B1 infections have been associated with the IAA-driven endotype of islet autoimmunity, but not with the GADA-first endotype, indicating potential heterogeneity in the triggers of the disease process (266). According

to the so-called polio hypothesis, the diabetogenic effect of enteroviruses is intensified in populations with a decreased rate of enteroviral infections such as in Finland (267). However, the convincing association between enteroviruses and type 1 diabetes might not be causal. To examine potential causality, a vaccine to prevent acute enteroviral infections and type 1 diabetes is under investigation and is among the most enticing strategies for the primary prevention of type 1 diabetes (258).

Gut microbiota

Since genetic factors alone do not explain the rapid increase of type 1 diabetes in young children, the immediate living environment, microbial contacts, and the effect of gut microbiome on the immune system have become fascinating candidates for playing a role in the pathogenesis of type 1 diabetes.

Early childhood is a unique period in several respects. First, during the early years the child's immune system undergoes fundamental education through numerous microbial contacts and infections. This is necessary in order to gain sufficient immunological memory and tolerance. Second, alongside the immunological development, the gut microbiome is stabilized by three years of age (268). Thereafter, the composition of the gut microbiome is similar to that of adults. Several factors modify the composition of the gut microbiome in infancy, including delivery mode, breastfeeding, introduction of solid foods, and use of antibiotics (269). The establishment of the gut microbiome is tightly related to the maturation of the immune system and might modulate intestinal immune responses (270). Third, simultaneously with these events, the first diabetes-associated autoantibodies usually appear. The use of antibiotics during the first four years is, however, not associated with islet autoimmunity (271).

Changes in the gut microbiome precede the appearance of islet autoimmunity. These include an increased relative abundance of the *Bacteroidetes* species and decreased abundance of the *Firmicutes* species and changes indicating a gut environment that favors inflammation (272, 273). In the prospective TEDDY study, children who progressed to type 1 diabetes demonstrated increased levels of *Bifidobacterium pseudocatenulatum*, *Roseburia hominis*, and *Alistipes shahii* in their intestinal microbiome (274). Intestinal impermeability is increased in children with signs of islet autoimmunity (275), and intestinal barrier function is decreased in type 1 diabetes (276).

It has been proposed that a chronic or repetitive dysbiosis of the intestinal microbiome might cause failure of the mechanisms sustaining self-tolerance and potentially lead to gut-initiated systemic immune responses. However, also favorable changes in the gut microbiota may occur since an increase in butyrate-producing bacteria could mediate a protective effect against islet autoimmunity (277). Early intake of oral probiotics has also shown a protective effect against type 1 diabetes among children with the high-risk *HLA-DR3/DR4* genotype (278). Altogether, these findings raise questions of whether changes in the gut microbiota and type 1 diabetes are linked causally.

Gut mycobiota

Fungi are present on all barriers of the human body, including the gut mucosa. Although knowledge of the role of fungi in health and disease is currently limited, the mycobiota might modify the function of the immune system and alter the composition of bacterial microbiota and virome, thereby modulating intestinal homeostasis (279). Fungal and bacterial dysbiosis and intestinal inflammation

appear to be associated with the development of type 1 diabetes in children with signs of beta-cell autoimmunity (280).

Gut virome

Virome sequencing techniques have enabled screening for all viruses simultaneously. In the DIPP study, no prominent changes were found in the gut virome prior to the signs of islet autoimmunity (281). However, an imbalance within the *Bacteroides* genus showed a potential association with islet autoimmunity, and a possible connection existed between *Bacteroides dorei* and the bacteriophage CrAssphage (282). The Australian Viruses in the Genetically at Risk (VIGR) study reported enrichment of enterovirus A species in the gut of children positive for islet autoantibodies (283). In the TEDDY study, a prolonged enterovirus B infection was associated with islet autoimmunity, although no association with the development of type 1 diabetes was found (265). In a study observing HLA-predisposed Finnish and Estonian children from birth, the gut virome in islet autoantibody-negative children showed higher diversity and higher abundance of *Circoviridae*-derived sequences than in autoantibody-positive children (284). The intestinal viruses might be capable of affecting the intestinal bacterial composition, thereby causing disturbance of intestinal homeostasis. The alterations in the gut virome linked to islet autoimmunity potentially may contribute to the development of type 1 diabetes.

Dietary factors

During the past decades the Western lifestyle, including the diet and eating habits, has changed fundamentally. Increased processing of foods, supplementation of foods, and awareness about the food's microbial composition have led to questions about whether dietary factors are behind the rapidly increasing incidence of type 1 diabetes.

Several aspects of the diet have been considered as potential contributors to islet autoimmunity and type 1 diabetes. The focus has been mainly on early diet in infancy, but also factors related to maternal diet during pregnancy and later food consumption in childhood have been studied.

Short breastfeeding has been suggested to increase the risk of islet autoimmunity. A meta-analysis showed a weak protective effect of breastfeeding against islet autoimmunity (285). Breastfed infants demonstrated a decreased risk for type 1 diabetes in a study combining two large Scandinavian birth cohorts compared with infants who had never been breastfed (286). During the introduction of gluten-containing cereals simultaneous breastfeeding was associated with protection against type 1 diabetes (287).

Early introduction of cow's milk proteins has also been investigated. In the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study, weaning to an extensively hydrolyzed cow's milk formula compared with a conventional formula did not reduce the risk of type 1 diabetes (288), although a smaller pilot study suggested a protective effect of weaning to a hydrolyzed formula against islet autoimmunity (289). However, high consumption of cow's milk later in childhood has been linked to increased risk of islet autoimmunity and type 1 diabetes, and genetic factors might modify this risk (290–292).

The risk of islet autoimmunity is potentially increased in association with both early (<4 months of age) and late (>6 to 9 months of age) introduction of solid foods, especially gluten (293–296).

However, also high intake of oats has been linked to increased risk of islet autoimmunity and type 1 diabetes (297).

Vitamin D may contribute to the development of type 1 diabetes. Nevertheless, the findings have been highly contradictory and the role of vitamin D in type 1 diabetes remains unclear (298). Higher serum 25-hydroxyvitamin D levels were associated with lower risk of islet autoimmunity in the TEDDY study (299). In a Norwegian study, increased levels of 25-hydroxyvitamin D in cord blood decreased the risk of type 1 diabetes depending on the vitamin D receptor genotype (300). However, in HLA-susceptible children in the DIPP study, no differences were seen in cord blood 25-hydroxyvitamin D concentrations between children who developed clinical diabetes or islet autoantibodies and those with no signs of beta-cell autoimmunity (301). Nor were any differences observed in the circulating concentrations of 25-hydroxyvitamin D between those who progressed to overt type 1 diabetes and matched controls in a longitudinal follow-up of DIPP participants (302). In the TRIGR ancillary study, serum 25-hydroxyvitamin D concentrations decreased months before the appearance of islet autoantibodies in children who later developed multiple autoantibodies or clinical type 1 diabetes (303). This was, however, considered to reflect the effects of vitamin D on the immune system, rather than a causal relationship between low vitamin D levels and type 1 diabetes.

Furthermore, a higher intake of omega-3 fatty acids, especially docosapentaenoic acid, has been inversely associated with the risk of islet autoimmunity (304). However, neither vitamin D nor omega-3 fatty acid supplementation during pregnancy have been shown to affect the risk of islet autoimmunity in offspring (305). Among other vitamins, high levels of plasma ascorbic acid might decrease the risk of islet autoimmunity (306). Also, the intake of probiotics during the first four weeks of life has been associated with a decreased risk of islet autoimmunity in infants carrying the high-risk *HLA-DR3-DQ2/DR4-DQ8* genotype (278). However, in a Finnish randomized controlled trial probiotic administration in infancy had no effect on the development of type 1 diabetes (307). Advanced glycation end-products and their receptors have been implicated in the early stages of islet autoimmunity (308).

Beta-cell stress

According to the accelerator hypothesis, insulin resistance or increased insulin demand caused by various factors, including rapid weight gain, increased height velocity, high body mass index (BMI), obesity, glucose overload, puberty, stressful life events, traumatic injuries, infections, steroid treatment, or psychological stress, may induce beta-cell stress and contribute to the pathogenesis of type 1 diabetes (309).

Childhood obesity has been linked to increased risk of later type 1 diabetes in several studies, including a meta-analysis (298, 310). Also high birthweight has been associated with increased risk of type 1 diabetes in a meta-analysis (311). Rapid weight gain in early life increases the risk of islet autoimmunity (312, 313). In the TEDDY study, a higher rate of weight gain during the slow growth phase after infancy increased the risk of progression from islet autoimmunity to clinical diabetes specifically among children with GADA as the primary autoantibody (313). Furthermore, high BMI might provoke the progression of islet autoimmunity to clinical diabetes (314).

Psychological stress is not to be overlooked. Children of parents who experienced a stressful life event during the child's first years or children who themselves encountered a serious life event have been reported to be at increased risk of type 1 diabetes (315, 316).

According to the overload hypothesis, environmental factors that cause excessive stress to beta cells might render them susceptible to immune destruction and apoptosis. Stress-inducing cytokine signals, viral infections, or metabolic overload may lead to impaired ER function and defective protein folding in beta cells, i.e. a cellular stress response known as the unfolded protein response (UPR). This might result in the release of improperly folded and potentially immunogenic peptide structures from the beta cell (317).

HETEROGENEITY IN TYPE 1 DIABETES

Observations on the rate of beta-cell destruction

The duration of the prediabetic period is highly variable, ranging from a couple of months to more than two decades (6). A notable increase in the pace of disease progression in recent years has raised concerns of whether the mechanisms that normally guard self-tolerance have weakened at the population level (2, 131, 148, 318). The age at diagnosis of type 1 diabetes has decreased, suggesting earlier and more aggressive autoimmune responses in children (2). In newly diagnosed patients with type 1 diabetes, the amount of residual beta-cell function is highly heterogeneous, indicating that the rate of functional beta-cell loss is also extremely variable (319).

IAA vs. GADA-first endotypes

Among the biochemically defined autoantibodies, IAA or GADA is usually the first to appear (151). The disease process beginning with either IAA or GADA shows different genetic and etiological associations and characteristics of islet autoimmunity. This suggests heterogeneity in the triggers of beta-cell autoimmunity.

As the primary autoantibody, IAA peak during the second year, whereas GADA usually appear later as the first autoantibody, at approximately 3–5 years of age, with a more even and broader profile (52, 117, 320). As the second autoantibody, GADA tend to appear soon after IAA (117). Initial IAA positivity has been associated with homozygosity for the *HLA-DR4-DQ8* haplotype, while GADA as the primary autoantibody has been linked to homozygosity for the *HLA-DR3-DQ2* haplotype (117, 126, 127, 320). Coxsackievirus B1 infections have been observed to be associated with the initiation of beta-cell autoimmunity in those with an IAA-initiated disease process, whereas no such association has been seen in those with initial GADA positivity (266). This implies that a diabetogenic Coxsackie B infection may be the trigger of beta-cell autoimmunity in the former cases.

Heterogeneity in cellular responses

Cytokine expression profiles in the peripheral blood of patients with newly diagnosed type 1 diabetes show notable differences in response to stimulation by different autoantigens (64). At diagnosis of type 1 diabetes, proinflammatory T-cell autoreactivity is more frequent in children and more often targeted to insulin and proinsulin peptides than in adult patients (321). In insulitis, two types of inflamed islets can be found, distinguished by the abundance of CD20-positive B cells in the insulitic

lesion (64). Several subtypes of type 1 diabetes also seem to exist depending on the level of the innate inflammatory activity (322). There is also disease stage-dependent variation in the immune phenotypes of T and B cells, indicating heterogeneity in cellular responses in type 1 diabetes (79).

Heterogeneity in metabolic condition at diagnosis

The metabolic status at diagnosis of type 1 diabetes is highly heterogeneous. In the TrialNet study, positivity for only one autoantibody, mostly GADA, was associated with insulin resistance and higher C-peptide levels relative to multiple autoantibody positivity (319). In the Finnish Pediatric Diabetes Register, more severe metabolic disturbance was seen among children with sporadic type 1 diabetes than in children from families with at least one member affected by type 1 diabetes (323). In the same study, children with a father affected by type 1 diabetes showed higher frequency of diabetic ketoacidosis and increased weight loss at diagnosis compared with offspring of mothers with diabetes (323). At diagnosis, girls presented with more severe metabolic deterioration than boys (324). Positivity for ZnT8A at diagnosis has been linked to older age and poorer metabolic state, including higher frequency of ketoacidosis (163). In general, age at diagnosis seems to have a substantial impact on the severity of type 1 diabetes (reviewed in 325).

PREDICTION AND PREVENTION OF TYPE 1 DIABETES

Prediction of type 1 diabetes

General aspects of disease prediction

The ability to precisely predict type 1 diabetes would enable cost-effective screening for risk of type 1 diabetes in the general population and facilitate the optimal design of intervention trials aimed at preventing or delaying the onset of clinical diabetes.

The focus on the prediction of type 1 diabetes has been on identifying risk individuals from the general population based on HLA risk genotypes or FDRs of individuals affected by type 1 diabetes and on measuring islet autoantibodies and metabolic markers of dysglycemia in such individuals. However, the disease process has proven to be highly heterogeneous (5). This has led to the consensus that the underlying causes of type 1 diabetes are complex and multifactorial and that novel biomarkers in addition to the traditional ones are needed for elaborate and correctly timed disease prediction.

The individuals at high disease risk can already be identified relatively reliably based on HLA risk genotypes and prediabetic autoantibody profiles. However, the factors determining the pace of the disease progression, the metabolic condition at diagnosis, and the disease progression after diagnosis are not yet completely understood. The goal of disease prediction is not only to gain knowledge of the natural history of type 1 diabetes, but to identify the heterogeneous subpopulations of at-risk individuals and to define the stages of disease progression. This might enable prevention trials suitable for both the disease stage and endotype to be conducted.

Although at present the screening for HLA risk genotypes and islet autoantibodies comprises the most reliable and easily accessible tools for the prediction of type 1 diabetes, autoantibodies only become measurable at a stage at which the immunological tolerance has already been exceeded and

the autoimmune response is ongoing. Thus, in the future it is crucial to generate biomarkers that are applicable before the signs of humoral autoimmunity emerge or that act as direct markers of beta-cell damage and/or dysfunction.

However, the prediabetic period *per se* provides a unique time frame for conducting secondary prevention trials. At this stage, it might still be possible to revert or halt the disease progression. Therefore, it is important to stratify the disease risk associated with the heterogeneous patterns of preclinical islet autoimmunity and to elucidate the role of autoantibody dynamics in detailed staging of the disease process. In this respect, prospective follow-up studies starting from birth or during the fetal period are essential since they might provide unique insights into the temporal events that occur at various stages of the disease process. Also, in primary prevention trials the islet autoantibodies comprise a valuable endpoint marker for initiated disease activity.

Predictive characteristics of islet autoantibodies

Islet autoantibodies have proven useful in the prediction of type 1 diabetes in children at increased genetic risk (326), in young FDRs of patients with type 1 diabetes (318), and in the pediatric general population (6). The most important findings in prospective studies of islet autoimmunity include the observation that beta-cell autoimmunity begins very early in life, with autoantibody seroconversions peaking during the first two years (51, 52). Young age at autoantibody appearance increases the risk of type 1 diabetes (8, 51, 52, 112). Another important finding has been the association of positivity for multiple autoantibodies with an extremely high risk of disease progression, with about 70% of multipositive children with *HLA-DR/DQ* risk genotypes and/or FDRs affected by type 1 diabetes progressing to clinical diabetes in 10 years (7, 149). Early and fast development of multipositivity is especially predictive of high disease risk (51, 149, 175).

As discussed, the prediabetic period is heterogeneous both in duration and in the context of the primary autoantigen (6, 117). The HLA genotype affects both the number and quality of islet autoantibodies during this period (327). The order of autoantibody appearance affects the disease risk (146). Furthermore, autoantibody titer and affinity and different autoantibody combinations increase the prognostic value of autoantibody testing when screening for risk of type 1 diabetes (8, 109, 188). Especially, rapid increase in autoantibody titers after seroconversion is predictive of fast progression to clinical disease (51). Also, the combination of persistent ICA and IAA positivity and high titers of ICA and IAA at seroconversion may predict rapid progression to overt disease (8, 51, 107, 108).

Although at present the screening for HLA risk haplotypes and islet autoantibodies comprises the most applicable tools for the evaluation of the risk for type 1 diabetes, autoantibodies are not very useful in predicting the timing of the clinical diagnosis. Other biomarkers might prove more precise in the prediction of the pace of the disease process. Moreover, there is still a need for improved understanding of the natural dynamics of islet autoantibodies in childhood to better discriminate non-progressive immunological activity from true progressive autoimmunity.

Factors related to progression rate

HLA gene effects on disease progression

Although HLA class II genes are strongly involved in the initiation of beta-cell autoimmunity, other genetic factors are likely to modify the rate of beta-cell destruction after the establishment of beta-cell autoimmunity (Table 2). Recent findings have strongly indicated that the HLA class II effect on disease progression is attenuated after the appearance of islet autoantibodies, but certain HLA class I alleles might modulate the rate of beta-cell destruction thereafter (201, 328, 329).

HLA class I allele *B*39* promotes disease progression after the appearance of the second autoantibody, and this risk-increasing effect is strongly enhanced among children carrying the high-risk *HLA-DR3/DR4* genotype (329). In the same study, the *HLA-A*03* allele was found to mediate a protective effect against disease progression in children without the high-risk *HLA-DR3/DR4* genotype (329). In another study, no effect of the *HLA-B*39* on disease progression was seen, but the *HLA-B*18* allele was associated with more rapid progression to clinical disease in children carrying the *HLA-DQ2* haplotype (328). *HLA-A*24* favored progression in *HLA-DQ8* carriers (328).

Among FDRs of patients with type 1 diabetes, the *HLA-A*24* allele was reported to mediate accelerated progression from multiple autoantibody positivity to clinical diabetes, but this association was detected only among relatives with the *HLA-DQ8* haplotype testing positive for IA-2A and/or ZnT8A (330). The *HLA-B*18* allele mediated more rapid progression to clinical disease in *HLA-DQ2* carriers positive for both GADA and IAA, but no effect was observed for the *HLA-B*39* allele (330). The disease-promoting *HLA-A*24* allele has been associated with attenuated islet autoantibody responses (331, 332). Also, the HLA class II haplotype *DRB1*15:01-DQA1*01:02-DQB1*06:02* has been shown to mediate a protective effect against disease progression in autoantibody-positive relatives of patients with type 1 diabetes at all stages of the disease process (333).

Non-HLA gene effects on disease progression

The effect of the predisposing SNP (rs689) in the *INS* gene is restricted to the early stages of islet autoimmunity (240). However, the predisposing SNP (rs2476601) in the *PTPN22* gene affects the progression rate to clinical disease also after the onset of humoral beta-cell autoimmunity (240). A predisposing SNP (rs3757247) in the *BACH2* gene affects the pace of disease progression specifically in children with IAA as the primary autoantibody (128). Similarly, predisposing SNPs (rs1701704, rs2292239) in the *IKZF4-ERBB3* region modify the progression rate only in children with GADA as the primary autoantibody (128). Among other non-HLA polymorphisms, the predisposing SNP (rs45450798) in the *PTPN2* gene affects the rate of beta-cell damage after seroconversion regardless of the primary autoantibody (128).

In the TEDDY study, a genetic risk score predicted the pace of disease progression (334). Also, another genetic risk score predicted the progression rate successfully among participants in the TrialNet study (222). In the DAISY study, a genetic risk score combining HLA and non-HLA genes improved the prediction of progression from islet autoimmunity to type 1 diabetes in children recruited from the general population (335). In the BABYDIAB study, several differences were found in the distributions of non-HLA genotypes between rapid and slow progressors to clinical type 1 diabetes (150). Furthermore, the predisposing SNP in the *SLC30A8* gene encoding ZnT8 might affect

the age at disease presentation, and accordingly, might modify the rate of disease progression (336). Progression to clinical disease is faster in ZnT8A-positive children homozygous for the *SLC30A8* SNP (rs1326634) alleles (159).

Autoantibody characteristics associated with progression rate

Several studies have reported findings on autoantibody characteristics related to the pace of disease progression. Development of multiple autoantibodies at a young age enhances the risk of progression to type 1 diabetes in the next 15 years (149). High initial levels of IAA increase the risk of rapid progression (51). In young children with multiple autoantibodies, characteristics that predict faster progression to clinical diabetes include young age and higher levels of IAA and IA-2A (153). Especially the appearance of IA-2A as the first biochemical autoantibody increases the risk of accelerated disease progression (117).

In the BABYDIAB study characterizing rapid and slow progressors to type 1 diabetes among multipositive individuals, the development of IA-2A was delayed among slow progressors (150). In the DAISY study, slow progressors were characterized by later onset of islet autoimmunity, less frequent positivity for IAA, and lower levels of autoantibodies, especially IAA, than rapid progressors (337). Also, the development of multiple autoantibodies was delayed among slow progressors (337). In a combined analysis of five prospective cohorts that attempted to identify the characteristics of slow progressors, GADA dominated as the most frequent autoantibody in the first sample positive for multiple autoantibodies among multipositive children who remained disease-free for over 10 years after initial seroconversion (338). Furthermore, in children with multiple autoantibodies recruited to the Type 1 Diabetes TrialNet, GADA positivity indicated reduced risk of progression to diabetes, while children positive for IA-2A were at increased risk (339).

Considering the IAA vs. GADA-first endotypes, the simultaneous appearance of two other autoantibodies with primary GADA positivity was reported to promote disease progression (340). However, no similar effect was observed in the case of primary IAA positivity (340). In children with IAA as the first autoantibody, IA-2A as the second reactivity predicted rapid disease progression, while no accelerating effect of GADA as the secondary autoantibody was seen after primary IAA positivity (340). In the DIPP study, no differences were found in the delay from seroconversion to diagnosis between children with either IAA or GADA as the primary autoantibody, but the disease progression was accelerated among children with IA-2A as the primary autoantibody and delayed among those with ZnT8A as the primary autoantibody (151). Early appearance of ZnT8A in the disease process has been associated with delayed disease progression (338).

In a subset of multipositive individuals, the loss of IAA positivity has been associated with a decreased rate of progression to clinical type 1 diabetes after 10 years of follow-up compared with persistent IAA positivity (194). In contrast, transient GADA positivity has been linked to more rapid progression in the presence of stable IAA and IA-2A (195).

Table 2. Factors related to progression rate to type 1 diabetes in the literature.

Characteristic associated with progression rate to T1D	Effect on disease progression after appearance of islet autoimmunity	Reference
HLA class II haplotype *DRB1*15:01-DQA1*01:02-DQB1*06:02*	Protective effect in autoantibody-positive FDRs of patients with T1D	333
HLA class I *B*39* allele	Promotes progression after appearance of secondary autoantibody, effect enhanced in individuals with *HLA-DR3/DR4* genotype	329
HLA class I *B*39* allele	No effect	328, 330
HLA class I *A*03* allele	Protective effect among individuals without the *HLA-DR3/DR4* genotype	329
HLA class I *B*18* allele	Rapid progression in *HLA-DQ2* carriers	328
HLA class I *B*18* allele	Accelerated progression from multiple autoantibody positivity to T1D diagnosis in GADA and/or IAA-positive FDRs of T1D patients with the *HLA-DQ2* haplotype	330
HLA class I *A*24* allele	Favors progression in *HLA-DQ8* carriers	328
HLA class I *A*24* allele	Accelerated progression from multiple autoantibody positivity to clinical T1D in IA-2A and/or ZnT8A-positive FDRs of T1D patients with the *HLA-DQ8* haplotype	330
INS gene SNP (rs689)	No effect	128, 240
PTPN22 gene SNP (rs2476601)	Promotes progression to clinical diabetes	240
PTPN2 gene SNP (rs45450798)	Promotes progression to clinical diabetes	128
IKZF4 gene SNP (rs1701704)	Promotes progression to clinical diabetes in individuals with GADA as the first autoantibody	128
ERBB3 gene SNP (rs2292239)	Promotes progression to clinical diabetes in individuals with GADA as the first autoantibody	128
BACH2 gene SNP (rs3757247)	Promotes progression to clinical diabetes in individuals with IAA as the first autoantibody	128
SLC30A8	Younger age at disease onset	336
SLC30A8 gene SNP (rs1326634) homozygosity in ZnT8A-positive children	Increases the risk of rapid progression	159
Genetic risk score combining *HLA* and non-*HLA* genes	Successfully predicted progression	335
Genetic risk score including predisposing non-*HLA* SNPs	Discriminative differences in distributions of non-*HLA* SNPs between rapid and slow progressors	150
Genetic risk score including predisposing non-*HLA* SNPs and *HLA* class I and class II alleles	Successfully predicted progression	222, 334
Multiple autoantibodies at a young age	Increases the risk of rapid progression	149, 153
IA-2A as the first autoantibody	Rapid progression	117, 151

Delayed development of IA-2A	Slower progression	150
High initial level of IA-2A	Increases the risk of progression	153
High initial level of IAA	Increases the risk of progression	51, 153
Lower initial level of IAA and other biochemical autoantibodies	Slow progression	337
Simultaneous appearance of two other autoantibodies in children with GADA as the primary autoantibody	Accelerated progression	340
ZnT8A as the first autoantibody	Delayed progression	151
Older age at appearance of islet autoimmunity	Slow progression	337
Slower development of multiple autoantibodies	Slow progression	337
GADA positivity in the first sample with multiple autoantibodies in multipositive children	Delayed progression	338
GADA positivity in children with two autoantibodies	Reduced risk of progression	339
IA-2A positivity in children with two autoantibodies	Increased risk of progression	339
Transient IAA positivity	Lower rate of progression	194
Transient GADA positivity	Accelerated progression in individuals with persistent IAA and IA-2A positivity	195
Higher BMI SDS	Accelerated progression	153
Reduced first-phase insulin response	Accelerated progression	153
Insulin resistance	No effect	153

T1D = type 1 diabetes. SDS = standard deviation score.

Early signs and biomarkers

Dysregulation of glucose metabolism

During the prediabetic disease process signs of metabolic dysregulation can be observed as the beta-cell damage proceeds (53). Reduced first-phase insulin response (FPIR) in the intravenous glucose tolerance test predicts progression to clinical diabetes in multipositive children (153). The FPIR declines rapidly during the last two years before symptomatic diabetes, but decreased responses may be observed as early as 4–6 years before diagnosis (341, 342).

Increasing levels of glycated hemoglobin (HbA1c) might improve the estimation of the timing of type 1 diabetes diagnosis. In the DAISY study, increasing HbA1c levels predicted increased risk of type 1 diabetes independently of random plasma glucose levels or the number of autoantibodies (343). In the TrialNet study, increased HbA1c values predicted the onset of clinical disease in the following years (344). In the DIPP series, an increase in HbA1c levels affected both the risk of diabetes and the delay from the observed rise in HbA1c to diagnosis (345). A consistent increase in HbA1c values was observed in progressors during the last two years before diagnosis.

During the oral glucose tolerance test (OGTT) both decreased early and increased late C-peptide responses indicate increased risk for type 1 diabetes (346). Similarly, impaired fasting glucose and IGT in OGTT predict progression to overt disease (347). Randomly obtained plasma glucose measurements might also provide a useful tool for disease prediction, although in the DAISY study an increase in random plasma glucose values predicted type 1 diabetes only marginally (HR 1.4) compared with an HR of 6.0 in the DIPP study (343, 347). Furthermore, continuous glucose monitoring has revealed increased glycemic variability in children with islet autoantibodies and predicts progression to clinical diabetes in prediabetic children (348).

How the preclinical changes in glucose metabolism relate temporally to the dynamics of islet autoantibodies is not completely understood. In the TrialNet study, the development of dysglycemia was associated with IA-2A titers (349). Moreover, insulin-like growth factors (IGFs) are involved in the regulation of glucose metabolism and might play a role in type 1 diabetes. Decreasing levels of especially IGF-1 have been observed preclinically in multipositive children and those who later progress to type 1 diabetes, and after the diagnosis the IGF-1 titers seem to decrease alongside the declining beta-cell function (350).

Proteomics

Studies on proteomics, metabolomics, and transcriptomics may provide information on the pathogenesis of type 1 diabetes before the appearance of humoral islet autoimmunity and might reveal novel biomarkers for the prediction of type 1 diabetes. Some of the recent findings in these fields are introduced below.

In general, age has a strong influence on the formation of the serum proteome (351). Among children progressing towards clinical type 1 diabetes, studies on the serum proteome have revealed an increase in the amount of proteins usually seen during acute inflammation, complement activation, and adaptive immune responses (352). Serum apolipoproteins M and C-IV have been reported to differ in levels between autoantibody-positive and -negative individuals (353). In children who later progress to type 1 diabetes, the expression of proteins involved in oxidative stress responses is increased already before autoantibody seroconversion compared with healthy controls (354).

Metabolomics

Metabolomic profiles might reflect changes in immunologic or metabolic status during the pathogenesis of type 1 diabetes. Dysregulation of lipid and amino acid metabolism in the fetal period might contribute to the process since several changes have been found in the cord blood lipidome of children who progress to diabetes at a young age, including reduced levels of choline-containing phospholipids (355, 356). A study on plasma metabolites in early infancy revealed that progression to clinical diabetes was associated with increased levels of methionine, but decreased abundance of amino acids, sugar metabolites, and fatty acids, including catabolites derived from microbial origin (357). The signs of humoral islet autoimmunity appeared to be associated with decreased glutamic and aspartic acids (357). In infancy, several plasma metabolites, including sphingomyelins, unsaturated phosphatidylcholines, phosphatidylethanolamines, glucosylceramides, and phospholipid ethers, have been inversely associated with the risk of developing multiple autoantibodies, whereas dicarboxylic acids might increase this risk (358). In the longitudinal TEDDY study, several changes

in plasma metabolites, including reduced plasma proline and branched-chain amino acids, preceded the appearance of the first islet autoantibody, and especially GABA showed an association with the IAA-first endotype (359). Furthermore, a longitudinal analysis of peripheral blood mononuclear cell metabolomics revealed that dysregulation of lipid metabolism in these cells was associated with progression to type 1 diabetes (360).

Transcriptomics

An analysis of transcriptomic profiles in the whole blood of children with signs of islet autoimmunity revealed dynamic changes in gene expression in pathways involved in the immune system (361). Gene expression profiles indicating immunological activation have been observed even prior to the signs of humoral beta-cell autoimmunity. Both in the Finnish DIPP study and in the German BABYDIET study, a type I interferon-inducible gene set was found to be transiently increased in expression before the appearance of islet autoantibodies in children with increased genetic risk for type 1 diabetes (362, 363). A recent study demonstrated a diminished amount of sulfatide in isolated pancreatic islets of patients with type 1 diabetes compared with healthy controls, indicating reduced expression of enzymes participating in sphingolipid metabolism (364). Changes in peripheral blood lymphocyte expression profiles may also prove useful in the assessment of risk for type 1 diabetes (365).

T-cell biomarkers

Although several mechanisms involving both innate and adaptive immune responses are likely to contribute to the pathogenesis of type 1 diabetes, the evidence so far refers to T cells as the primary mediators (73). Certain T-cell subsets might be useful as biomarkers of treatment efficacy in clinical trials (71, 366). Follicular and peripheral T helper cells are increased in number before and at diagnosis of type 1 diabetes and might prove useful as biomarkers for disease prediction (75, 77, 78). Also functional deficiencies, altered cytokine signaling, and changes in transcriptional profiles of FOXP3 regulatory T cells have been associated with type 1 diabetes and might provide another promising approach for the prediction of type 1 diabetes (80, 82, 367–369).

Prevention of type 1 diabetes

The globally rising incidence of type 1 diabetes calls for effective preventive measures (1). No cure for type 1 diabetes will probably be found before successful prevention. The timeline for prevention of type 1 diabetes can be staged into four categories: 1) primary prevention before onset of islet autoimmunity, 2) secondary prevention before diagnosis of type 1 diabetes, 3) tertiary prevention in newly diagnosed patients to preserve residual β-cell function, 4) and late prevention of secondary complications in established type 1 diabetes and attempts to revive lost β-cell function.

The JDRF's global vision is to discover a primary prevention approach safe enough to be administered to the general childhood population without the evaluation of disease risk (370). A lead candidate for such a measure would be a preventive vaccine for early immunization (370).

To date, prevention trials carried out in human populations have demonstrated only limited success in achieving their goal (371). Some past and ongoing trials are summarized in Table 3. The

most promising results have been achieved in studies of teplizumab (71), rituximab (56), alefacept (72), abatacept (366), and anti-thymocyte globulin (372). Also, a transient benefit after the administration of oral insulin was seen in a subgroup of individuals with high IAA levels in the Diabetes Prevention Trial-Type 1 trial (373).

The main obstacles to successful prevention of type 1 diabetes derive from the incomplete understanding of disease heterogeneity and pathomechanisms and the lack of biomarkers for cost-effective screening. Improved biomarkers are needed to target appropriate interventions to suitable populations (371). Some beneficial effects in the past trials may not have been observed due to suboptimal study design (371). Considering the optimal selection of the study population, the disease stage, and the applied intervention in prevention trials, the dilemma exists that in the early stages of the disease process more effective interventions may be used, but the disease prediction is poorer and a larger population is predisposed to potential side effects of the treatment. In contrast, in later stages, the disease prediction is more precise, but applicable interventions are less effective due to the already advanced disease process (374).

Consideration of these aspects in future trial design might improve the probability of observing a beneficial effect. The use of combination therapies targeting multiple facets of the disease pathogenesis might generate better results than a single-target therapy alone. The key to achieving sufficient resources for carrying out successful prevention trials is an active collaboration globally. Shorter and more affordable pilot studies should be encouraged to identify the best intervention candidates for larger clinical trials (371). The primary focus in intervention trials should, however, always be on clinically meaningful improvements. Even mild preservation of the beta-cell function is a victory for patients living with type 1 diabetes.

Ethical issues on disease prediction and prevention

Delay of onset of type 1 diabetes might have meaningful positive effects on the social, emotional, mental, and physical development of young patients *en route* to type 1 diabetes. Delayed onset of the clinical disease could provide young individuals more disease-free time in their teenage years, during which the compliance to treatment is often poor due to challenges of insulin therapy, and glycemic control is often affected by this (375). Life expectancy is the lowest among patients who have been diagnosed at a young age, emphasizing the importance of at least delaying the onset of type 1 diabetes (3, 4). Young age at diagnosis might also increase the lifetime risk of secondary complications due to the longer disease duration (3).

Children participating in prospective follow-up studies are diagnosed at an earlier and metabolically less severe stage of the disease pathogenesis than the background population, probably due to the increased education of the families about the symptoms of type 1 diabetes (376). Increased awareness of the symptoms in general might promote earlier contact with healthcare.

On the other hand, the screening for risk of type 1 diabetes raises a range of ethical concerns. Awareness of the disease risk might cause anxiety and psychological discomfort in study participants and their families (377). However, enrollment in follow-up studies might also decrease parental stress and improve quality of life and glycemic control in participants affected by type 1 diabetes (378, 379). In general, parents prefer to be informed about the high risk of type 1 diabetes in their offspring, even if no preventive measure is available (380). In prospective studies, screening for genetic risk of newborn infants occurs often in the days immediately following birth, which is a sensitive period for

mothers psychologically, especially concerning the wellbeing and health of their babies. Increased maternal anxiety is a common response to the information that the child is at genetic risk of diabetes (381). However, the increased anxiety usually dissipates over time (377).

As an essential part of screening and prevention strategies in follow-up studies, a communication protocol might be considered to avoid misunderstandings and potential psychological harm to study participants and their families. This also requires good communication skills and emotional sensitivity of the study personnel to detect any ethical concerns that may arise. Behavior rating scales for the assessment of parental attitudes might prove helpful in targeting parent education efforts in studies screening for risk of type 1 diabetes in pediatric populations (382).

Table 3. Some previous and ongoing trials aimed at preventing type 1 diabetes.

Study	Intervention	Stage	Outcome achieved
TRIGR	Hydrolyzed casein formula	Primary	No
BABYDIET	Gluten-free diet during first year	Primary	No
DIPP	Intranasal insulin	Secondary	No
DPT-1	Oral insulin	Secondary	Transient benefit in subgroup with high IAA levels
DPT-1	Parenteral insulin	Secondary	No
Belgian Diabetes Registry	Subcutaneous insulin	Secondary	No
INIT-II	Intranasal insulin	Secondary	Ongoing
TrialNet	Oral insulin	Secondary	No
Pre-POINT	Oral/intranasal insulin	Primary	Yes (immune response to insulin)
DIAPREV-IT	Alum-GAD (Diamyd ®)	Secondary	No
DIAPREV-IT2	Alum-GAD + Vitamin D3 2000 IU/d	Secondary	Ongoing
TrialNet	Teplizumab (anti-CD3 monoclonal antibody)	Secondary	Yes
TrialNet	Abatacept	Tertiary	Yes
TrialNet NIP	Docosa-hexaenoic acid	Primary	No
ENDIT	Nicotinamide (vitamin B3)	Secondary	No
DENIS	Nicotinamide	Secondary	No
TrialNet, AIDA	Canakinumab	Tertiary	No
TrialNet	GAD vaccine	Tertiary	No
TrialNet	Mycophenolate mofetil/daclizumab	Tertiary	No
TrialNet	Rituximab	Tertiary	No long-term effect
DEFEND-1	Otelixizumab	Tertiary	No
T1DAL	Alefacept	Tertiary	Yes
TrialNet	Anti-thymocyte globulin	Tertiary	Yes
TrialNet	Hydroxychloroquine	Secondary	Ongoing
University of Buffalo, USA	Etanercept	Tertiary	Yes (pilot)

AIMS OF THE STUDY

This thesis focuses on assessing the dynamics of islet autoantibodies in childhood, from birth up to 15 years of age, and on identifying the demographic, genetic, and immunological characteristics of rapid and slow progression to clinical type 1 diabetes in children with increased HLA-conferred risk for type 1 diabetes recruited from the general population.

The main aims of the thesis were as follows:

1. To determine the genetic, immunological, and demographic characteristics of rapid disease progression from seroconversion to autoantibody positivity.

2. To determine the genetic, immunological, and demographic characteristics of slow disease progression from seroconversion to autoantibody positivity.

3. To assess the seroconversion dynamics of individual islet autoantibodies during prospective follow-up from birth up to 15 years of age.

4. To evaluate the predictive value of autoantibody testing for clinical type 1 diabetes and to describe systematically for the first time the role of ZnT8A in this context.

5. To examine the role of primary autoantibodies in disease progression.

6. To assess the role of inverse seroconversions and autoantibody fluctuations in disease progression.

7. To examine the value of ICA in the prediction of type 1 diabetes.

SUBJECTS AND METHODS

Study subjects

Type 1 Diabetes Prediction and Prevention (DIPP) Study

The DIPP study is a prospective Finnish general population-based birth cohort study carried out in the university hospitals of Turku, Oulu, and Tampere. The DIPP study aims at monitoring the signs of humoral islet autoimmunity and progression to clinical type 1 diabetes among individuals with increased HLA-conferred risk for type 1 diabetes and at identifying measures for preventing or delaying the manifestation of clinical type 1 diabetes in at-risk individuals. The recruitment was launched in Turku University Hospital in 1994, in Oulu University Hospital in 1995, and in Tampere University Hospital in 1997 and is ongoing.

Newborn infants born in the participating hospitals are screened for HLA risk genotypes from cord blood. Families with an infant carrying an eligible genotype are invited to participate in the study follow-up starting from the age of 3 months (383). Islet autoantibodies are analyzed as markers of beta-cell autoimmunity from venous blood samples obtained at regular clinical visits. In Oulu and Tampere, the children are seen at the ages of 3, 6, 12, 18, and 24 months and annually thereafter, and in Turku every 3 months up to the age of 2 years and thereafter every 6 months. Children testing positive for autoantibodies are invited to visits every 3 months. According to the DIPP study design, the visits continue up to the diagnosis of type 1 diabetes or to the age of 15 years. Children with multiple autoantibodies may continue in the follow-up after the age of 15 years. Efforts are made to minimize the dropout rate during the follow-up. Infants with severe congenital abnormalities or disease are excluded from the study. Families with no common language (i.e. Finnish, Swedish, or English) with the study personnel are also excluded.

The current DIPP study cohorts were recruited in 1994–2003. At the time, the three hospitals together covered a population of approximately 1.2 million (24% of the total population in Finland), and the number of annual births in these hospitals was around 11 000 (20% of the total number of annual births in Finland). Between 1994 and 1998, more than 90% of the babies born annually in these hospitals took part in the HLA screening (383). Thereafter, the proportion decreased gradually to an estimated 80%. By the end of July 1997, written informed consent had been obtained for 12 170 newborns, all of whom underwent HLA screening (112). By the end of July 2003, altogether 75 813 infants had attended the HLA screening (8).

Publication I

In Study I, the first 1006 children (53% boys) recruited to the DIPP study in 1994–1997 were followed from birth up to 15.5 years of age for the development of islet autoantibodies and for progression to clinical type 1 diabetes (Table 4). Follow-up visits after the age of 14.5 years were considered as the final study visits. Venous blood samples obtained before the age of 15.5 years were included in the detailed autoantibody analyses. Altogether 542 children (55.8%) completed the 15-year follow-up. In Study I, all available samples from the 1006 children were analyzed for ICA, IAA, GADA, IA-2A, and ZnT8A.

By the age of 15.5 years, 35 children (3.5%) had progressed to type 1 diabetes. Among the progressors, two had their first autoantibody-positive sample at diagnosis and were therefore excluded from the autoantibody analyses. Both children had dropped out from the active follow-up several years before the diagnosis (3.6 and 10.4 years). Autoantibody data at diagnosis were available for 34 progressors. In Study I, autoantibody titers were compared between progressors and multiple autoantibody-positive controls matched for the closest date of birth (+/- 3 months), DIPP study center, HLA risk genotype, and sex.

Publications II and III

To identify the characteristics of rapid and slow progression from seroconversion to type 1 diabetes, 7410 HLA-predisposed children (52.6% boys) recruited to the DIPP study between November 1994 and July 2003 were followed from birth until the end of December 2015 (Table 4). The children in the study cohort had attended the DIPP follow-up visits for at least one year before the end of 2003 or had been diagnosed with type 1 diabetes before the age of one year by the end of 2003. The first 1006 children included in Study I were also included in Studies II and III.

Among the 7410 children, 13 (0.2%) had developed clinical type 1 diabetes without any detectable autoantibody positivity and were therefore excluded from the autoantibody analyses. Twelve of the 13 children had a long time (3–12 years) since the last autoantibody sampling before diagnosis, which might have contributed to the absence of detectable autoantibodies. Fourteen children (0.2%) had their first autoantibody-positive sample at the diagnosis of type 1 diabetes. Since the delay from seroconversion to diagnosis could not be defined in these children, they were excluded from the autoantibody analyses in Studies II and III.

Methods

Genetic screening

Analyses of HLA genotypes

Screening for the major type 1 diabetes-risk associated *HLA-DR/DQ* haplotypes from cord blood was performed by using polymerase chain reaction (PCR) amplification, followed by time-resolved fluorometry with lanthanide-labeled allele sequence-specific oligonucleotide probes, as previously described (384, 385). The risk conferred by some of the HLA class II genotypes is shown in Table 1.

Analyses of non-HLA genotypes

Non-HLA SNPs in Studies II and III were analyzed by using the Sequenom (San Diego, California, USA) platform at the Genome Center of Eastern Finland, University of Eastern Finland, Kuopio, Finland. The SNP marker analysis was considered successful if the proportion of failed SNP analyses remained below 10% (386). According to the original research setting, SNP markers were analyzed from DIPP study participants who had tested positive for ICA and for at least one additional biochemical autoantibody (IAA, GADA, and/or IA-2A) and from their autoantibody-negative controls (two controls per case) matched for the closest date of birth, sex, and study center (128).

Non-HLA SNPs predisposing to type 1 diabetes were selected for the current analysis by using ImmunoBase, a web-based resource providing information on the genetics of immune-mediated diseases in man (www.immunobase.org, formerly www.t1database.org). In Study II, predisposing SNPs of 25 non-HLA genes were analyzed to assess their potential association with rapid disease progression (Table 5). In Study III, additional SNPs were included and altogether 45 non-HLA SNPs were investigated for associations with delay from seroconversion to diagnosis among progressors, age at seroconversion, age at diagnosis of type 1 diabetes, delay from single to multiple autoantibody positivity, and disease-free time from seroconversion in children with confirmed autoantibody positivity (Table 5).

Table 4. DIPP study cohorts in Studies I–III. Continuous variables are medians (range). *One progressor was excluded from the autoantibody analyses in Study III since only one autoantibody-positive sample was available at the age of 3 months. In this case, maternal autoantibody positivity could not be excluded.

	Publication I DIPP cohort (n=1006)	Publications II and III DIPP cohort (n=7410)
DIPP study center	Turku, Oulu	Turku, Oulu, Tampere
Recruitment	Born between November 1994 and July 1997	Followed for ≥one year or progressed to T1D before the age of one year by the end of 2003
Follow-up period in the current study	Until the age of 15.5 years	Until 31 December 2015
Timing of study visits	Every 3–12 months from birth	Every 3–12 months from birth
Clinical characteristic		
Sex, boys	533 (53.0%)	3895 (52.6%)
High-risk *HLA-DQB1*02/*03:02* genotype	252 (25.0%)	1575 (21.3%)
Progression to type 1 diabetes	35 (3.5%)	Study II 248 (3.3%) Study III 247 (3.3%)*
Follow-up time from birth including progressors, years	14.9 (1.9–15.5)	16.2 (0.9–21.1)
Follow-up time from birth excluding progressors, years	15.0 (1.2–15.5)	NA
Age at diagnosis of type 1 diabetes, years	8.0 (2.1–14.2)	7.6 (0.9–18.0)
Age at seroconversion, years	7.4 (0.3–15.1)	5.0 (0.2–15.1)
Time from seroconversion to diagnosis, years	3.8 (0.1–11.1)	4.0 (0.02–17.0)

Table 5. Investigated non-HLA SNPs in Studies II and III.

Chromosome	SNP	Gene	Minor / major allele	Risk genotype	Reference	Study
1p13.2	rs2476601	*PTPN22*	A/G	AA, AG	237	II, III
11p15.5	rs689	*INS*	T/A	AA	211	II, III
10p15.1	rs12722495	*IL2RA*	G/A	AA, AG	216	II, III
10p15.1	rs2104286	*IL2RA*	G/A	AA, AG	386	II, III
12q13.2	rs1701704	*IKZF4*	C/A	CC, AC	214	II, III
12q13.2	rs2292239	*ERBB3*	A/C	AA, AC	210	II, III
1q31.2	rs2816316	*RGS1*	G/T	TT	216	II, III
2q33.2	rs3087243	*CTLA4*	A/G	GG, AG	210	II, III
4q27	rs17388568	*ADAD1/IL2*	A/G	AA, AG	210	II, III
18q11.2	rs45450798	*PTPN2*	C/G	GG, CG	216	II, III
2q24.2	rs1990760	*IFIH1*	C/T	TT, CT	210	II, III
6q15	rs3757247	*BACH2*	A/G	AA, AG (only GADA+)	129, **212	II, III
12q24.1	rs3184504	*SH2B3*	A/G (T/C)	AA, AG (TT, CT)	210	II, III
15q25.1	rs3825932	*CTSH*	C/T	TT	212	II, III
16p13.1	rs12708716	*CLEC16A*	G/A	AA, AG	210	II, III
19q13.4	rs601338	*FUT2*	A/G	AA (non-secretor)	220	II, III
1p31.1 (non-confirmed)	rs630115	*LOC646538*	A/G	GG, AG	***217, 386	II, III
15q14	rs17574546	*RASGRP1*	C/A	CC, AC	213	II, III
10p11.2	rs2666236	*NRP1*	A/G (T/C)	AA, AG (TT, CT)	210	II, III
2q32.2	rs7574865	*STAT4*	T/G	TT, GT	213	II, III
18q22.2	rs763361	*CD226*	T/C	TT, CT	210	II, III
1q32.1	rs3024505	*IL10*	T/C	CC, CT	208	II, III
3p21.3	rs11711054	*CCR3-CCR5*	G/A	GG, AG	208, 216	II, III
12p13.3	rs3764021	*CLEC2D*	T/C (A/G)	CC (GG)	210	II, III
6q23.3	rs6920220	*TNFAIP3*	A/G	AA, AG	221	II
16p13	rs2903692	*KIAA0350 /CLEC16A*	A/G	GG, AG	217	III
Chr 10	rs11594656	*IL2RA/CD25*	A/T	TT, AT	210	III
11p15.5	rs3842729	*INS*	C/T	CC	236	III
Chr 8	rs13266634	*SLC30A8*	C/T	CC, TT homozygotes (ZnT8A+)	159	III
12q24	rs17696736	*NAA25*	G/A	GG, AG	210	III
Chr 2	rs2111485	*IFIH1*	A/G	GG, AG	218, 221	III
Chr 7	rs6965571	*GIMAP5*	A/G	GG, AG	387	III

Chr 7	rs2286899	*GIMAP5*	C/T	TT, CT*	387	III
Chr 7	rs10361	*GIMAP5*	C/G	GG, CG*	387	III
Chr 7	rs2293174	*GIMAP4*	C/A	AA, AC*	387	III
Chr 7	rs2373816	*GIMAP4*	G/A	GG, AG*	387	III
Chr 3	rs4402960	*IGF2BP2*	G/T	GG risk genotype for T2D*	388	III
4q27	rs4505848	*KIAA1109/IL2*	A/G	susceptible locus	208	III
Chr 10	rs41295061	*IL2RA*	C/A	CC, AC	221	III
12p13.31	rs4763879	*CD69*	G/A	AA, AG	208	III
1p13.2	rs6679677	*PHTF1*	C/A	AA, AC	210	III
2q12.1	rs917997	*IL18RAP*	A/G	GG, AG	216	III
2p13	rs6546909	*DQX1*	T/A	AA, AT	210	III
2q11	rs9653442	*AFF3-LOC150577*	G/A (C/T)	GG, AG (CC, CT)	210	III
10p15.1	rs11258747	*PRKCQ*	T/G	TT	208	III
13q32	rs9585056	*GPR183/EBI2*	C/T	CC, CT	219	III

*No association with type 1 diabetes reported. **Association with type 1 diabetes reported for another *BACH2* gene SNP (rs11755527) in high linkage disequilibrium with the investigated rs3757247 SNP (r^2=0.94). ***Association with type 1 diabetes reported for another *LOC646538* gene SNP (rs672797) in complete linkage disequilibrium with the investigated rs630115 SNP (r^2=1).

Autoantibody analyses

The analyses of type 1 diabetes-associated autoantibodies were carried out in the Research Laboratory, Department of Pediatrics, University of Oulu, Oulu, Finland, except in the case of ZnT8A, which were analyzed in the PEDIA Laboratory, University of Helsinki, Helsinki, Finland. The primary screening in the DIPP study was ICA-based from 1994 to 2002. Three biochemical autoantibodies, IAA, GADA, and IA-2A, were analyzed from all earlier and subsequent samples of study participants who tested positive for ICA or were diagnosed with clinical type 1 diabetes. All samples from participants born in 2003 onwards were analyzed directly for ICA, IAA, GADA, and IA-2A. Furthermore, all samples obtained from the first 1006 DIPP children born in 1994–1997 were analyzed directly for ICA, IAA, GADA, and IA-2A, and subsequently also for ZnT8A.

ICA were analyzed by using a standardized indirect immunofluorescence method on sections of frozen human blood group O donor pancreas (16). The ICA titers were expressed in JDF units (JDFU). The detection limit for ICA positivity was 2.5 JDFU. The biochemical autoantibodies IAA, GADA, IA-2A, and ZnT8A were analyzed by using specific radiobinding assays, as previously described (154, 164, 389–391). Autoantibody titers of the biochemical autoantibodies were expressed in relative units (RU), reflecting the specific binding of autoantibodies to their antigens. The RU were based on a standard curve run on each assay plate by using the MultiCalc software (PerkinElmer Life Sciences-Wallac, Inc., Turku, Finland). Cut-off values for autoantibody positivity were set at the 99th

percentile levels in 370–374 non-diabetic Finnish children (IAA, 3.48 RU; GADA 5.36 RU; IA-2A, 0.43 RU; ZnT8A, 0.61 RU). Before analyses, all serum samples were stored at –70°C. All samples with autoantibody titers between the 97th and 99.5th percentile values of the reference population were reanalyzed to confirm the result.

According to the results of the Diabetes Autoantibody Standardization Program (DASP) and the Islet Autoantibody Standardization Program (IASP) between 2010 and 2016, the sensitivities of the IAA, GADA, IA-2A, and ZnT8A radiobinding assays were 36–62%, 64–88%, 62–72%, and 62–70%, respectively. The corresponding specificities were 94–98%, 94–99%, 93–100%, and 99–100%.

Definitions

In Studies I and III, confirmed autoantibody positivity was defined as positivity for at least one autoantibody in at least two consecutive samples before diagnosis of type 1 diabetes. In Study II, confirmed seroconversion was defined as positivity for at least one autoantibody in at least two samples, and also five children who had been diagnosed with classical type 1 diabetes, but had tested autoantibody-positive in only one sample before the diagnosis were considered as autoantibody-positive progressors.

The date of seroconversion to autoantibody positivity was defined as the date of draw of the first autoantibody-positive sample. Multipositivity was defined as positivity for two or more autoantibodies in the same sample. Primary autoantibody was defined as the first-appearing single autoantibody. Autoantibody titers in all studies were compared among individuals positive for the specific autoantibody reactivity. Inverse seroconversion was defined as becoming permanently autoantibody-negative after testing autoantibody-positive. Fluctuating autoantibody positivity was defined as one or more autoantibody-negative samples between positive samples. In the DIPP study, infants with transplacentally transferred maternal autoantibodies were excluded from the autoantibody analyses if no *de novo* synthesis of islet autoantibodies was detected. Progressors were defined as participants who were diagnosed with type 1 diabetes.

Type 1 diabetes was diagnosed according to the World Health Organization criteria (392). Whenever possible, the date of diagnosis of type 1 diabetes was verified by using the data of the Finnish Pediatric Diabetes Register, which covers over 90% of children under the age of 16 years diagnosed with type 1 diabetes in Finland since 2002. An autoantibody sample was considered to be obtained at the time of diagnosis if it was drawn within two weeks before or after the date of diagnosis. In the DIPP study, information on the affected FDRs of the study children was attained by using structured questionnaires completed by the parents shortly after the birth of the index child.

Progression to type 1 diabetes was considered to be rapid if type 1 diabetes was diagnosed within 1.5 years (18 months) from seroconversion to autoantibody positivity. The rationale for this definition was to identify approximately one-fifth of the study population with the most rapid disease progression. Although there was no biological evidence to support the selected cut-off, this definition was chosen based on practical aspects, for instance, that the individuals with aggressive islet autoimmunity would likely comprise one of the main target groups for future preventive interventions. In this regard, we considered that it takes a couple of months to confirm autoantibody positivity, exclude clinical type 1 diabetes, and recruit the child to a clinical trial. On the other hand, if a preventive treatment is successful, the effect will become apparent in a short period of time.

Disease progression was considered as slow in cases where the delay from seroconversion to diagnosis lasted for at least 7.26 years (87 months). This cut-off was set at the 75th percentile value of the delay to diagnosis observed among progressors in the DIPP study cohort. Again, no biological evidence supports the selected cut-off, but this definition provided a favorable setting to investigate the factors related to a considerably longer delay from seroconversion to diagnosis than seen among most progressors in the DIPP study cohort. As the definitions of rapid and slow progression were partly data-driven, the generalizability of the results might be limited.

In Study III, multipositive non-progressors were defined as participants with multiple autoantibodies who had been monitored for at least 7.26 years after seroconversion to autoantibody positivity, but had remained unaffected by type 1 diabetes by 31 December 2015 (n=198).

Data management and statistical methods

IBM SPSS predictive analytics software for Macintosh (versions 22.0 and 25.0, IBM Corp., Armonk, NY, USA) was applied for statistical analyses. The confidence interval (CI) was set at 95% and the statistical significance at $P<0.05$ (two-tailed). To test statistical differences, cross-tabulation, Pearson's χ^2 test, Fisher's exact test, the Mann-Whitney U test, and the Kruskall-Wallis test were used, when applicable. Sensitivity and specificity values were calculated as previously described (393). Survival distributions were analyzed by using the log-rank test (Kaplan-Meier analysis). In Study I, survival tables were created by applying the GraphPad Prism 8 Software for Mac Os X. In Study II, Cox regression analyses were applied to identify independent variables predictive of rapid progression to type 1 diabetes.

In the SNP association analyses, the high possibility of false-positive associations caused by multiple statistical testing was taken into consideration. In Study II, the *P*-values of the multiple comparisons were corrected by using the Bonferroni correction. In Study III, the false discovery rate was controlled by using the Benjamini-Hochberg step-up procedure applying the cumulative minimum principle (R Software for Statistical Computing for Macintosh, version 3.3.3, R Foundation, Vienna, Austria) (394). Corrections for multiple comparisons were not applied in the data analysis in Study I due to the overly conservative nature of the Bonferroni correction. The observed associations were, however, interpreted cautiously.

Ethical considerations

The DIPP study protocol was approved by the local ethics committees of the participating hospitals. The study has been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. The legal guardians of the newborn infants gave written informed consent for HLA screening and for participation in the DIPP study follow-up.

RESULTS

Dynamics of islet autoantibodies from birth up to 15 years of age

In Study I, the 1006 HLA-predisposed children, including progressors to type 1 diabetes, were followed from birth over a median time of 14.9 years (range 1.9–15.5 years) (Table 4). In total, 542 participants (55.8%) completed the 15-year follow-up. By 15.5 years of age, type 1 diabetes was diagnosed in 35 children (3.5%), and confirmed seroconversion to autoantibody positivity was detected in 275 children (27.3%), including 32 progressors.

Age-related dynamics of islet autoantibodies

Over the first 15 years, there were marked differences in the age-related behavior of individual autoantibodies. The overall seroconversion rate accelerated towards puberty, mostly due to low-titer ICA seroconversions. The cumulative proportions of all autoantibodies increased up to 15 years of age (Table 6). There was no difference in the overall seroconversion rate between boys and girls with increasing age (Table 7). To examine the dynamics of islet autoantibodies, we calculated seroconversion rates of each autoantibody reactivity in four age periods (Table 6). The low-titer ICA seroconversion rate increased continuously, while the seroconversion rates of biochemically defined autoantibodies and high-titer ICA decreased with advancing age. In children under the age of 3 years, IAA prevailed as the most common autoantibody, but after 3 years of age, ICA exceeded IAA as the predominant autoantibody (Figure 2).

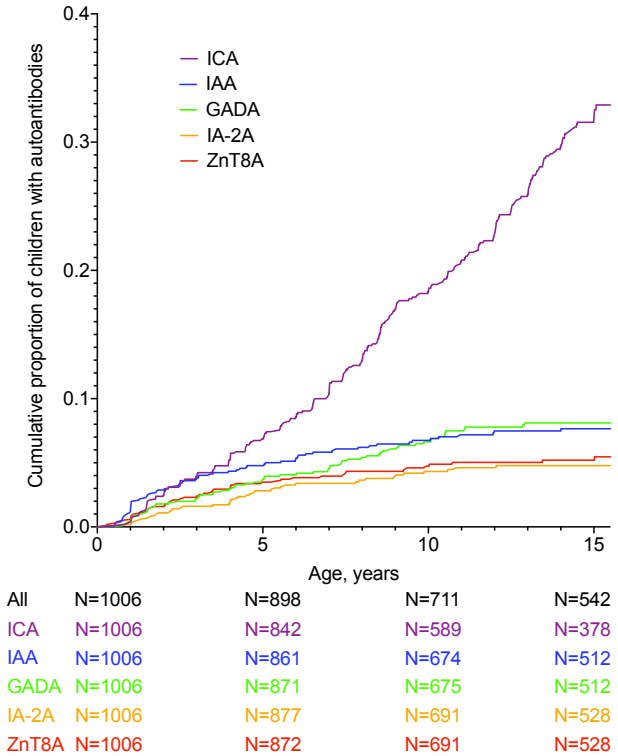

Figure 2. Development of ICA, IAA, GADA, IA-2A, and ZnT8A by 15.5 years of age.

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Table 6. Cumulative frequencies of autoantibodies by the age of 15.5 years, median age at seroconversion, and seroconversion rates in four age periods (0–1.99, 2–4.99, 5–9.99, and 10–15.5 years) among the 1006 HLA-susceptible children in Study I.
JDFU=Juvenile Diabetes Foundation units. Reproduced with permission from *J Clin Endocrinol Metab*.

Autoantibody type	Cumulative frequency of autoantibody positivity at 15.5 years (%)	Median age at seroconversion, years (range)	Seroconversions per 100 follow-up years			
			0–1.99 years	2–4.99 years	5–9.99 years	10–15.5 years
ICA, ≥2.5 JDFU	245 (24.4)	8.1 (0.5–15.1)	1.2	1.5	2.3	2.8
ICA, ≥10 JDFU	72 (7.2)	4.2 (0.5–14.5)	1.1	0.6	0.5	0.4
IAA	69 (6.9)	2.5 (0.5–14.0)	1.4	0.6	0.4	0.2
GADA	69 (6.9)	5.0 (0.8–12.9)	0.9	0.6	0.6	0.3
IA-2A	42 (4.2)	4.1 (1.0–12.1)	0.5	0.6	0.3	0.1
ZnT8A	48 (4.8)	3.1 (0.3–15.0)	0.8	0.6	0.3	0.1
At least 1 autoantibody	275 (27.3)	7.4 (0.3–15.1)	2.0	1.8	2.5	2.7
At least 1 autoantibody, ICA ≥10 JDFU	126 (12.5)	4.0 (0.3–14.5)	2.0	1.2	0.9	0.5
At least 1 biochemical autoantibody	113 (11.2)	4.0 (0.3–14.0)	1.7	1.1	0.9	0.5
Multiple (≥2) autoantibodies	71 (7.1)	4.0 (0.8–14.4)	1.1	0.6	0.5	0.4
Multiple biochemical autoantibodies	50 (5.0)	3.0 (0.8–12.1)	1.0	0.5	0.3	0.1

Table 7. Seroconversion rate to autoantibody positivity in boys vs. girls in the age periods of 0–4.99, 5–9.99, and 10–15.5 years.

Sex	Cumulative frequency of autoantibody positivity at 15.5 years	*P*-value	Age at seroconversion, years	*P*-value	Seroconversions per 100 follow-up years			*P*-value
	N (%)		Median (range)		0–4.99 years	5–9.99 years	10–15.5 years	
	With ICA							
Boys	146 (27.4)	0.97	7.3 (0.3–15.0)	0.96	1.0	1.3	1.5	0.92
Girls	129 (27.3)		7.4 (0.3–15.1)		0.9	1.2	1.2	
	Without ICA							
Boys	70 (13.1)	0.04	5.0 (0.3–14.0)	0.10	0.7	0.6	0.4	0.22
Girls	43 (9.1)		3.5 (0.3–12.0)		0.6	0.3	0.1	

Development of multipositivity

The cumulative proportion of multipositive children grew steadily up to 15 years of age, but only four children turned positive for multiple biochemical autoantibodies after the age of 10 years (Figure 3).

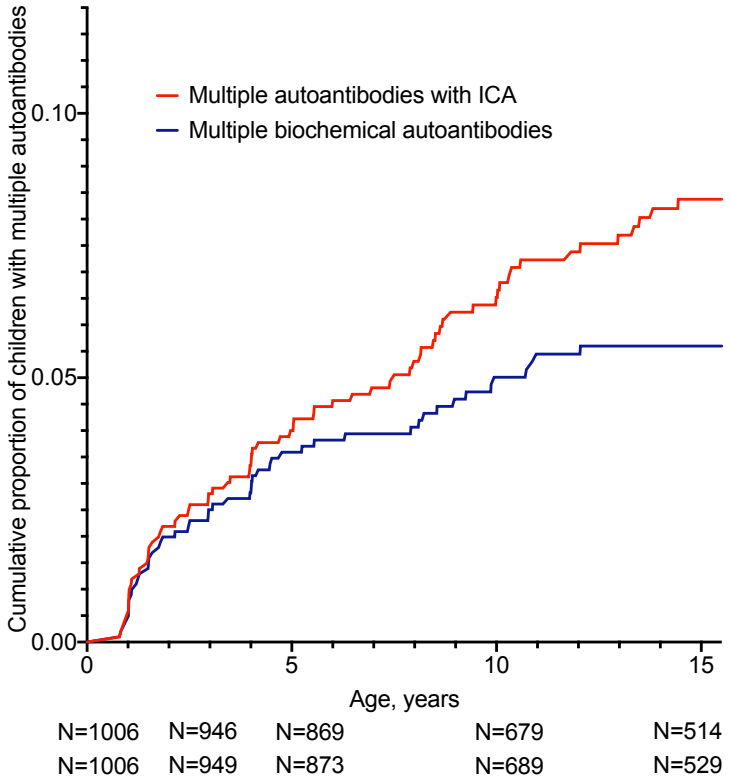

Figure 3. Development of multiple autoantibodies by the age of 15.5 years with and without ICA in the analysis. Reproduced with permission from *J Clin Endocrinol Metab*.

Effect of HLA genotype on islet autoimmunity

By 15 years of age, 8.3% of children with the high-risk *HLA-DQB1*02/*03:02* genotype and 1.9% of those with moderate-risk *HLA-DQB1*03:02/x* (x≠**02*, **03:01*, **06:02*) genotypes had progressed to type 1 diabetes. At 15 years, the proportions of children in the HLA risk groups remained virtually unaltered, with 136 children (25.1%) in the high-risk and 406 children (74.9%) in the moderate-risk HLA group (Table 4). The high-risk genotype increased the risk of islet autoimmunity compared with moderate-risk genotypes (36% vs. 24%; $P<0.001$; screening without ICA 18% vs. 9%; $P<0.001$). Among the seroconverted children, the high-risk HLA genotype was associated with an increased frequency of IAA (33% vs. 21%; $P=0.03$), GADA (36% vs. 20%; $P=0.003$), and multiple autoantibodies (35% vs. 21%; $P=0.01$; screening without ICA 29% vs. 13%; $P=0.002$), but was not significantly associated with positivity for ICA (90% vs. 89%; $P=0.70$), IA-2A (21% vs. 13%; $P=0.07$), or ZnT8A (23% vs. 15%; $P=0.08$) compared with moderate-risk genotypes.

Primary autoantibodies in different age groups

The primary autoantibody profiles at initial seroconversion showed unique characteristics in different age groups. In young children, IAA and ZnT8A appeared commonly as the first autoantibody, but at preschool age IA-2A- and GADA-initiated islet autoimmunity became common. ICA as the single first autoantibody increased towards puberty (Figure 4). Multipositive seroconversions occurred most frequently in the youngest age groups. The primary ICA seroconversion rate accelerated continuously up to the age of 15 years, but primary seroconversions positive for biochemical autoantibodies peaked already at a young age. The only exception was seen in the case of primary GADA seroconversion rate, which continued to remain stable throughout childhood (Table 8).

Primary autoantibodies at seroconversion in progressors and non-progressors

Consistently with the age-associated profiles, the primary autoantibodies at seroconversion showed conspicuous differences between progressors and non-progressors (Table 9). In progressors, the primary autoantibody was in most cases either IAA or ZnT8A among those who were not multipositive at seroconversion, whereas in multipositive non-progressors it was mostly GADA. Accordingly, GADA as the single first autoantibody was rarely seen among progressors. This was considered to reflect the fact that ICA might overlap with positivity for the biochemical autoantibodies. Therefore, the analysis was also carried out without ICA. However, even when ICA was excluded from the analysis, primary GADA positivity remained rare among progressors, but was associated with multipositive non-progressors (Table 9). Notably, ICA as the single primary autoantibody at seroconversion implied non-progressive autoimmunity regardless of the ICA titer.

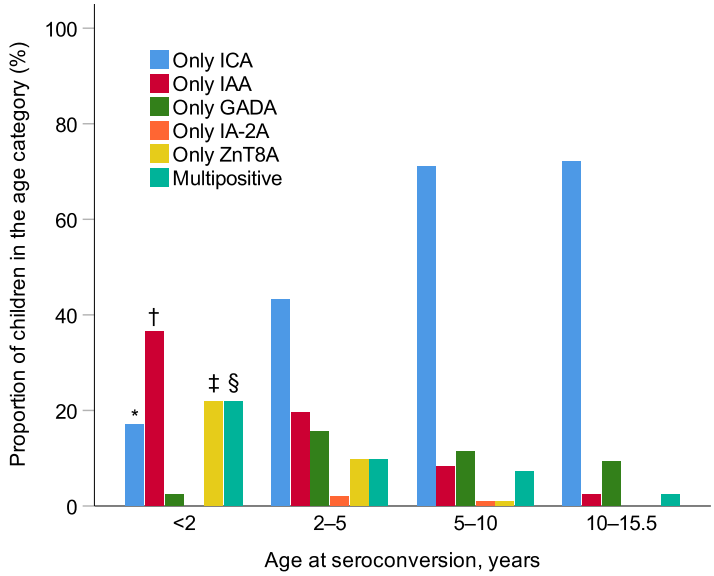

Figure 4. Autoantibody profiles at initial seroconversion in the age groups 0–2 (N=41, a), 2–5 (N=51, b), 5–10 (N=97, c), and 10–15.5 (N=86, d) years. **P*<0.05 compared with all other groups, †*P*<0.05 a vs. b, a vs. c, a vs. d, b vs. c, b vs. d, ‡*P*<0.05 a vs. b, a vs. c, a vs. d, b vs. c, §*P*<0.05 a vs. c, a vs. d. Reproduced with permission from *J Clin Endocrinol Metab*.

Table 8. Autoantibodies at primary seroconversion and the rates of primary seroconversions positive for each autoantibody in four age periods with ICA (A) and without ICA (B) in the analysis.

A

With ICA	N (% of primary seroconversions in the age period)[b]			
Autoantibody positivity	0–1.99 years	2–4.99 years	5–9.99 years	10–15.5 years
ICA	10 (24.4)	25 (49.0)	75 (77.3)	76 (88.4)
IAA	24 (58.5)	14 (27.5)	10 (10.4)	2 (2.3)
GADA	8 (19.5)	11 (21.6)	15 (15.5)	9 (10.5)
IA-2A	1 (2.4)	3 (5.9)	3 (3.1)	1 (1.2)
ZnT8A[a]	11 (35.5)	9 (18.4)	2 (3.0)	0 (0.0)
Multiple (≥2) AABs	9 (22.0)	5 (9.8)	7 (7.2)	2 (2.3)
Multiple biochemical AABs	7 (17.1)	5 (9.8)	1 (1.0)	0 (0.0)
	Primary seroconversions per 100 follow-up years*			
	0–1.99 years	2–4.99 years	5–9.99 years	10–15.5 years
ICA	0.5	0.9	1.9	2.4
IAA	1.2	0.5	0.3	0.1
GADA	0.4	0.4	0.4	0.3
IA-2A	0.0	0.1	0.1	0.0
ZnT8A	0.5	0.3	0.1	0.0
Multiple (≥2) AABs	0.4	0.2	0.2	0.1
Multiple biochemical AABs	0.3	0.2	0.0	0.0

B

Without ICA	N (% of primary seroconversions in the age period)[d]			
Autoantibody positivity	0–1.99 years	2–4.99 years	5–9.99 years	10–15.5 years
IAA	24 (70.6)	14 (46.7)	12 (35.3)	3 (20.0)
GADA	8 (23.5)	11 (36.7)	18 (52.9)	10 (66.7)
IA-2A	1 (2.9)	4 (13.3)	3 (8.8)	1 (6.7)
ZnT8A[c]	11 (45.8)	9 (32.1)	3 (11.5)	1 (20.0)
Multiple biochemical AABs	7 (20.6)	5 (16.7)	1 (2.9)	0 (0.0)
	Primary seroconversions per 100 follow-up years*			
	0–1.99 years	2–4.99 years	5–9.99 years	10–15.5 years
IAA	1.2	0.5	0.3	0.1
GADA	0.4	0.4	0.5	0.3
IA-2A	0.0	0.1	0.1	0.0
ZnT8A	0.5	0.3	0.1	0.0
Multiple biochemical AABs	0.3	0.2	0.0	0.0

[a]ZnT8A data available at primary seroconversion for 159 children.
[b]Primary seroconversions in the age period 0–1.99 years, *n*=41; 2–4.99 years, *n*=51; 5–9.99 years, *n*=97; 10–15.5 years, *n*=86.
[c]ZnT8A data available at primary biochemical autoantibody seroconversion for 83 children.
[d]Primary seroconversions without ICA in the age period 0–1.99 years, *n*=34; 2–4.99 years, *n*=30; 5–9.99 years, *n*=34; 10–15.5 years, *n*=15.
*Primary seroconversions positive for autoantibody reactivity per 100 follow-up years.
AAB=autoantibody. Reproduced with permission from *J Clin Endocrinol Metab*.

Table 9. Primary autoantibody signatures in progressors, multipositive non-progressors, and single autoantibody-positive non-progressors with and without ICA in the analysis.

Primary autoantibodies	Total	Progressors	Multipositive non-progressors	Single AAB-positive non-progressors	*P**	*P*†	*P*‡	*P*§
With ICA	*n*=275	*n*=32	*n*=39	*n*=204				
	N (%)							
Only ICA[a]	172 (62.5)	1 (3.1)	8 (20.5)	163 (79.9)	NS	***	***	***
Only IAA[a]	35 (12.7)	6 (18.8)	9 (23.1)	20 (9.8)	NS	***	**	**
Only GADA[a]	28 (10.2)	0 (0)	13 (33.3)	15 (7.4)	**	NS	***	NS
Only IA-2A[a]	2 (0.7)	0 (0)	0 (0)	2 (1.0)	NA	NA	NA	NS
Only ZnT8A[a,b]	15 (5.5)	7 (21.9)	4 (10.3)	4 (2.0)	**	***	NS	***
Multiple autoantibodies	23 (8.4)	18 (56.3)	5 (12.8)	0 (0)	***	NA	NA	***
Without ICA	*n*=113	*n*=32	*n*=19	*n*=62				
	N (%)							
Only IAA[a]	41 (36.3)	8 (25.0)	5 (26.3)	28 (45.2)	NS	NS	NS	NS
Only GADA[a]	37 (32.7)	3 (9.4)	9 (47.4)	25 (40.3)	*	*	NS	*
Only IA-2A[a]	5 (4.4)	2 (6.3)	0 (0)	3 (4.8)	NS	NS	NS	NS
Only ZnT8A[a,c]	17 (15.0)	8 (25.0)	3 (15.8)	6 (9.7)	NS	*	NS	*
Multiple autoantibodies	13 (11.5)	11 (34.4)	2 (10.5)	0 (0)	NS	NA	NA	***

*P**= Progressors vs. multipositive non-progressors

P†= Progressors vs. single autoantibody-positive non-progressors

P‡= Multipositive vs. single autoantibody-positive non-progressors

P§= Progressors vs. all non-progressors

[a]Multipositive individuals were excluded from the comparisons of single autoantibody-positive signatures between the groups.

[b]ZnT8A data available at seroconversion for 27 progressors, 35 multipositive non-progressors, and 97 single autoantibody-positive non-progressors. Among these children, 12 progressors and 30 multipositive non-progressors had only one autoantibody at initial seroconversion.

[c]ZnT8A data available at biochemical autoantibody seroconversion for 28 progressors, 18 multipositive non-progressors, and 37 single autoantibody-positive non-progressors. Among these children, 18 progressors and 16 multipositive non-progressors had only one biochemical autoantibody at initial seroconversion.

**P*<0.05

***P*<0.01

****P*<0.001

NS=non-significant. Reproduced with permission from *J Clin Endocrinol Metab*.

Inverse seroconversions and fluctuating autoantibodies

Among the islet autoantibodies, IAA experienced most frequently an inverse seroconversion and demonstrated most often fluctuations in autoantibody positivity (Table 10). Inverse seroconversions of IAA, IA-2A, and ICA were less common among progressors than non-progressors. No reversions or fluctuations of ICA occurred in progressors. None of the children with multiple biochemical autoantibodies reverted back to complete autoantibody negativity, but one of these children tested positive for only ICA in the last available sample.

Table 10. Inverse seroconversions and autoantibody fluctuations in Study I.

	Total* (n=275)	Progressors (n=32)	Autoantibody-positive non-progressors (n=243)	*P*-value
	N (%)			
Inverse seroconversions	124 (45.1)	13 (40.6)	111 (45.7)	NS
Fluctuating AABs	110 (40.0)	12 (37.5)	98 (40.3)	NS
Multiple fluctuations of the same reactivity	49 (17.8)	8 (25.0)	41 (16.9)	NS
Inverse seroconversions and/or fluctuations	180 (65.5)	21 (65.6)	159 (65.4)	NS
Inverse seroconversions and fluctuations	54 (19.6)	4 (12.5)	50 (20.6)	NS
	N (%)[a]			
Inverse seroconversion				
ICA	77 (31.4)	0 (0)	77 (36.2)	<0.001
IAA	34 (49.3)	7 (25.9)	27 (64.3)	0.002
GADA	20 (29.0)	5 (20.0)	15 (34.1)	NS
IA-2A	4 (9.3)	0 (0)	4 (22.2)	0.01
ZnT8A	9 (18.8)	3 (12.0)	6 (26.1)	NS
Fluctuating positivity				
ICA	69 (28.2)	0 (0)	69 (32.4)	<0.001
IAA	25 (36.2)	7 (25.9)	18 (42.9)	NS
GADA	14 (20.3)	1 (4.0)	13 (29.5)	0.01
IA-2A	6 (14.0)	2 (8.0)	4 (22.2)	NS
ZnT8A	8 (16.7)	6 (24.0)	2 (8.7)	NS

*Total number of seroconverters.
[a]Proportion of participants positive for the specific autoantibody reactivity.
AAB=autoantibody. NS=non-significant.
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Effect of autoantibody titers on autoantibody persistence

As IAA and ICA demonstrated the most unstable characteristics among the islet autoantibodies, we examined the effect of their titers on the rate of inverse seroconversions. For each child positive for IAA and/or ICA, the peak autoantibody titer of each reactivity was selected for analysis. The highest IAA titers were significantly higher among children with persistent IAA positivity than among those who became negative for IAA (median 34.6 vs. 11.0 RU; $P<0.001$). The peak ICA titers also reached higher levels among those who remained ICA-positive than among those who reverted back to ICA negativity (median 10.0 vs. 5.0 JDFU; $P<0.001$).

Persistence of insulin autoantibodies and progression rate to type 1 diabetes

We examined the association of autoantibody persistence with the pace of disease progression. Compared with progressors with persistent IAA positivity, those who became IAA-negative before diagnosis had a prolonged delay from seroconversion to diagnosis (8.2 vs. 3.4 years; $P=0.01$). At diagnosis, the proportion of IAA-positive children was higher among those with the high-risk HLA genotype than among those with moderate-risk genotypes (75% vs. 36%; $P=0.02$). Children who tested IAA-positive at diagnosis were diagnosed at a younger age than those who tested IAA-negative (median 6.6 vs. 11.7 years; $P=0.02$), although the age at seroconversion did not differ between these groups (1.2 vs. 1.0 years; $P=0.42$). No significant associations emerged between positivity for other islet autoantibodies and the age at diagnosis (data not shown). Data on autoantibodies at diagnosis of type 1 diabetes are shown in Table 11.

Table 11. Autoantibody positivity and median titers for positive autoantibodies at diagnosis of type 1 diabetes among the 34 progressors with available autoantibody samples at diagnosis.

	Autoantibody type	N (%)
Autoantibody positivity	ICA	33 (97.1)
	IAA	20 (58.8)
	GADA	22 (64.7)
	IA-2A	29 (85.3)
	ZnT8A	19 (65.5)
	Multipositivity	33 (97.1)
	Multiple biochemical autoantibodies	28 (82.4)
		Median (range)
Autoantibody titers	ICA, JDFU	44.0 (3.0–640.0)
	IAA, RU	18.9 (4.0–303.0)
	GADA, RU	34.0 (5.9–165.7)
	IA-2A, RU	91.1 (0.4–126.7)
	ZnT8A, RU	4.3 (0.7–17.0)

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Dynamics of islet autoimmunity and season of birth

In Study I, season of birth contributed to age-related characteristics of islet autoimmunity. Multipositivity for biochemical autoantibodies developed more often before the age of 2 years among children born in the winter, from December to February, than among those born in other seasons (67% vs. 25%; *P*=0.004). Multipositivity for biochemical autoantibodies tended to appear also at a younger age among children born in the winter than among those born in other seasons (median 1.5 vs. 4.0 years; *P*=0.06). However, being born in the winter did not increase the frequency of progression to type 1 diabetes compared with being born in other seasons (4% vs. 3%; *P*=0.43). Inverse seroconversions to IAA showed a higher frequency among children born in the fall, from September to November, than among those born in other seasons (77% vs. 43%; *P*=0.03). However, these observations should be interpreted cautiously, as the number of participants in the comparisons was small and significant differences in the distributions may have arisen by chance.

Comparison of progressors to multiple autoantibody-positive non-progressors

As multipositivity is a strong predictor of type 1 diabetes within 15 years, we examined the profiles of progressors compared with multipositive non-progressors (149). Multipositivity within a year from seroconversion predicted strongly progression to clinical disease (Table 12). However, this difference did not reach statistical significance when ICA was excluded from the analysis. All progressors and multipositive non-progressors developed ICA positivity.

When ICA was omitted from the analysis, only a few differences were seen between the groups, but those that remained statistically significant included a higher frequency of the high-risk HLA genotype and younger age at ZnT8A positivity among the progressors than among the multipositive non-progressors. The progressors tested also significantly more often positive for IAA at seroconversion before the age of 5 years than the multipositive non-progressors.

No inverse seroconversions or fluctuations of ICA occurred among progressors, but the corresponding proportions among multipositive non-progressors were notable (Table 13). Without ICA in the analysis, IAA positivity disappeared more often among multipositive non-progressors than progressors. Furthermore, progressors and multipositive non-progressors demonstrated significant differences in the dynamics of autoantibody titers. Compared with matched multipositive controls, the titers of ICA, IA-2A, and ZnT8A started to increase in progressors already months before the diagnosis (Table 14).

Table 12. Clinical characteristics in progressors and multipositive non-progressors with ICA (A) and without ICA (B) in the analysis.
RU=relative units. JDFU=Juvenile Diabetes Foundation units. **P*<0.05, ***P*<0.01, ****P*<0.001, NS=non-significant. Reproduced with permission from *J Clin Endocrinol Metab*.

A

	Progressors (n=32)	Multipositive non-progressors (n=39)	*P*-value
	N (%)		
HLA genotype			
High-risk HLA genotype	20 (62.5)	12 (30.8)	**
Sex			
Boys	18 (56.3)	28 (71.8)	NS
Affected FDRs at birth	5 (15.6)	2 (5.1)	NS
Period of seroconversion			NS
0–5 years	25 (78.1)	21 (53.8)	
5–10 years	6 (18.8)	15 (38.5)	
10–15.49 years	1 (3.1)	3 (7.7)	
AAB positivity at seroconversion			
ICA	12 (37.5)	11 (28.2)	NS
IAA	19 (59.4)	11 (28.2)	**
GADA	11 (34.4)	17 (43.6)	NS
IA-2A	6 (18.8)	0 (0)	**
ZnT8A	12 (44.4)	6 (17.1)	*
Multipositivity	18 (56.3)	5 (12.8)	***
Multipositivity without ICA	11 (34.4)	2 (5.1)	**
AAB positivity during follow-up			
ICA	32 (100)	39 (100)	NA
IAA	27 (84.4)	21 (53.8)	**
GADA	25 (78.1)	27 (69.2)	NS
IA-2A	24 (75.0)	16 (41.0)	**
ZnT8A	25 (78.1)	18 (46.2)	**
Multipositivity	32 (100)	39 (100)	NA
Multipositivity within a year from seroconversion	30 (93.8)	19 (48.7)	***
Multipositivity ≤5 years of age	25 (78.1)	14 (35.9)	***
Multipositivity without ICA	31 (96.9)	19 (48.7)	***
	Median (range)		
Age at seroconversion, years	1.7 (0.3–10.6)	4.2 (0.3–12.9)	**
ICA	2.1 (0.8–10.6)	5.5 (1.0–14.4)	***
IAA	1.8 (0.5–10.3)	3.0 (0.8–12.0)	NS
GADA	1.8 (0.8–9.9)	5.5 (0.8–12.9)	*
IA-2A	4.0 (1.0–10.6)	5.0 (1.2–12.1)	NS
ZnT8A	2.2 (0.3–10.7)	5.0 (0.3–15.0)	*
Multipositivity	2.0 (0.8–10.6)	7.4 (1.0–14.4)	***
Multipositivity without ICA	2.5 (0.8–10.7)	4.0 (1.0–12.1)	NS
AAB titers at seroconversion			
ICA, JDFU	13.5 (4.0–320.0)	5.0 (4.0–28.0)	**
IAA, RU	9.8 (5.0–56.1)	7.1 (4.1–19.0)	*
GADA, RU	28.0 (6.5–176.3)	11.0 (5.7–65.7)	NS
IA-2A, RU	12.6 (1.4–109.9)	–	NA
ZnT8A, RU	1.7 (0.7–2.8)	0.7 (0.7–7.5)	NS
Time from single to multiple autoantibody positivity, years	0.5 (0.3-2.7)	1.5 (0.2-9.4)	**

B

	Progressors (n=32)	Non-progressors with multiple biochemical autoantibodies (n=19)	*P*-value
	N (%)		
HLA genotype			
High-risk HLA genotype	20 (62.5)	6 (31.6)	*
Sex			
Boys	18 (56.3)	13 (68.4)	NS
Affected FDRs at birth	5 (15.6)	1 (5.3)	NS
Period of seroconversion			NS
0–5 years	25 (78.1)	15 (78.9)	
5–10 years	6 (18.8)	2 (10.5)	
10–15.49 years	1 (3.1)	2 (10.5)	
Period of seroconversion			NS
0–2 years	17 (53.1)	8 (42.1)	
2–5 years	8 (25.0)	7 (36.8)	
5–10 years	6 (18.8)	2 (10.5)	
10–15.49 years	1 (3.1)	2 (10.5)	
AAB positivity at seroconversion			
IAA	19 (59.4)	6 (31.6)	NS
GADA	11 (34.4)	11 (57.9)	NS
IA-2A	6 (18.8)	0 (0)	NS
ZnT8A	13 (46.4)	5 (27.8)	NS
Multipositivity	11 (34.4)	2 (10.5)	NS
AAB positivity at seroconversion at age ≤5 years			
IAA	18 (72.0)	6 (40.0)	*
GADA	8 (32.0)	7 (46.7)	NS
IA-2A	3 (12.0)	0 (0)	NS
ZnT8A	11 (52.4)	5 (33.3)	NS
Multipositivity	10 (40.0)	2 (13.3)	NS
AAB positivity during follow-up			
IAA	27 (84.4)	13 (68.4)	NS
GADA	25 (78.1)	18 (94.7)	NS
IA-2A	24 (75.0)	15 (78.9)	NS
ZnT8A	25 (78.1)	16 (84.2)	NS
Multipositivity	31 (96.9)	19 (100)	NS
Multipositivity within a year from seroconversion	24 (77.4)	11 (57.9)	NS
Multipositivity ≤5 years of age	23 (74.2)	12 (63.2)	NS
	Median (range)		
Age at seroconversion, years	1.6 (0.3–10.6)	2.5 (0.3–10.5)	NS
IAA	1.8 (0.5–10.3)	1.6 (0.8–11.0)	NS
GADA	1.8 (0.8–9.9)	3.9 (0.8–10.5)	NS
IA-2A	4.0 (1.0–10.6)	5.4 (1.2–12.1)	NS
ZnT8A	2.2 (0.3–10.7)	4.0 (0.3–15.0)	*
Multipositivity	2.5 (0.8–10.7)	4.0 (1.0–12.1)	NS
AAB titers at seroconversion			
IAA, RU	9.8 (5.0–56.1)	7.7 (4.1–8.8)	NS
GADA, RU	28.0 (6.5–176.3)	31.3 (5.7–65.7)	NS
IA-2A, RU	12.6 (1.4–109.9)	–	NA
ZnT8A, RU	1.5 (0.7–2.8)	0.7 (0.7–7.5)	NS
Time from single to multiple autoantibody positivity, years	0.5 (0.1-4.0)	0.9 (0.2-7.4)	NS

Table 13. Inverse seroconversions and fluctuations of autoantibodies in progressors and multipositive non-progressors with ICA (A) and without ICA (B) in the analysis.

A

	Progressors (n=32)	Multipositive non-progressors (n=39)	*P*-value
	N (%)[a]		
Inverse seroconversions			
ICA	0 (0)	8 (20.5)	**
IAA	7 (25.9)	11 (52.4)	NS
GADA	5 (20.0)	4 (14.8)	NS
IA-2A	0 (0)	2 (12.5)	NS
ZnT8A	3 (12.0)	3 (16.7)	NS
Fluctuating autoantibodies			
ICA	0 (0)	9 (23.1)	**
IAA	7 (25.9)	12 (57.1)	*
GADA	1 (4.0)	6 (22.2)	NS
IA-2A	2 (8.0)	3 (18.8)	NS
ZnT8A	6 (24.0)	1 (5.6)	NS

B

	Progressors (n=32)	Non-progressors with multiple biochemical autoantibodies (n=19)	*P*-value
	N (%)[a]		
Inverse seroconversions			
IAA	7 (25.9)	8 (61.5)	*
GADA	5 (20.0)	1 (5.6)	NS
IA-2A	0 (0)	1 (6.7)	NS
ZnT8A	3 (12.0)	3 (18.8)	NS
Fluctuating autoantibodies			
IAA	7 (25.9)	7 (53.8)	NS
GADA	1 (4.0)	4 (22.2)	NS
IA-2A	2 (8.3)	3 (20.0)	NS
ZnT8A	6 (24.0)	1 (6.3)	NS

[a]Proportion among those who tested positive for the autoantibody reactivity.
*P<0.05
**P<0.01
***P<0.001
NS=non-significant.
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Table 14. Median autoantibody titers in progressors (*n*=20) and multipositive controls (non-progressors who developed multiple autoantibodies by the age of 15.5 years, *n*=20) matched for date of birth (+/- 3 months), DIPP study center, HLA risk genotype, and sex.

		Progressors	Multipositive controls	*P*-value
		Median (range)		
In the latest study sample available within a year from seroconversion				
	Age, years	2.6 (1.5–11.5)	2.7 (1.4–11.4)	NS
	ICA, JDFU	31.0 (0.0–130.0)	0.0 (0.0–640.0)	***
	IAA, RU	7.8 (0.0–294.8)	5.3 (0.0–10.2)	*
	GADA, RU	8.8 (0.0–151.7)	0.1 (0.0–165.1)	**
	IA-2A, RU	1.0 (0.1–122.3)	0.1 (0.0–18.7)	**
	ZnT8A, RU	1.3 (0.1–5.1)	0.2 (0.0–37.9)	**
In the latest study sample available 18–24 months before diagnosis of type 1 diabetes				
	Age, years	6.5 (0.5–12.5)	6.4 (0.6–12.5)	NS
	ICA, JDFU	66.5 (0.0–640.0)	2.5 (0.0–191.0)	**
	IAA, RU	5.3 (0.0–76.9)	5.3 (0.4–30.3)	NS
	GADA, RU	10.0 (0.1–141.1)	0.2 (0.1–1489.5)	NS
	IA-2A, RU	77.9 (0.1–116.6)	0.1 (0.0–93.5)	***
	ZnT8A, RU	3.2 (0.1–22.5)	0.3 (0.0–35.7)	***
In the latest study sample available 6–12 months before diagnosis of type 1 diabetes				
	Age, years	7.6 (1.5–13.6)	7.6 (1.7–13.6)	NS
	ICA, JDFU	60.5 (4.0–640.0)	4.5 (0.0–380.0)	***
	IAA, RU	4.9 (0.2–97.5)	4.1 (0.0–11.1)	NS
	GADA, RU	22.3 (0.1–146.1)	1.8 (0.1–173.7)	NS
	IA-2A, RU	72.7 (0.0–142.6)	0.1 (0.0–96.2)	**
	ZnT8A, RU	2.9 (0.1–14.6)	0.2 (0.0–5.7)	***

RU=relative units. JDFU=Juvenile Diabetes Foundation units. Reproduced with permission from *J Clin Endocrinol Metab*.
*$P<0.05$
**$P<0.01$
***$P<0.001$
NS=non-significant.

General observations on progression rate

In Studies II and III, we aimed at identifying factors associated with the progression rate from seroconversion to type 1 diabetes. In the study cohort comprising 7410 HLA-predisposed children, the median duration of the prediabetic period was 4.0 (range 0.02–17.0) years. The distribution of the delay from seroconversion to diagnosis did not correlate with age at seroconversion (Spearman correlation coefficient 0.051; *P*=0.43), and there was no evidence of a naturally occurring cut-off between rapidly and slowly progressing forms of islet autoimmunity (Figure 5).

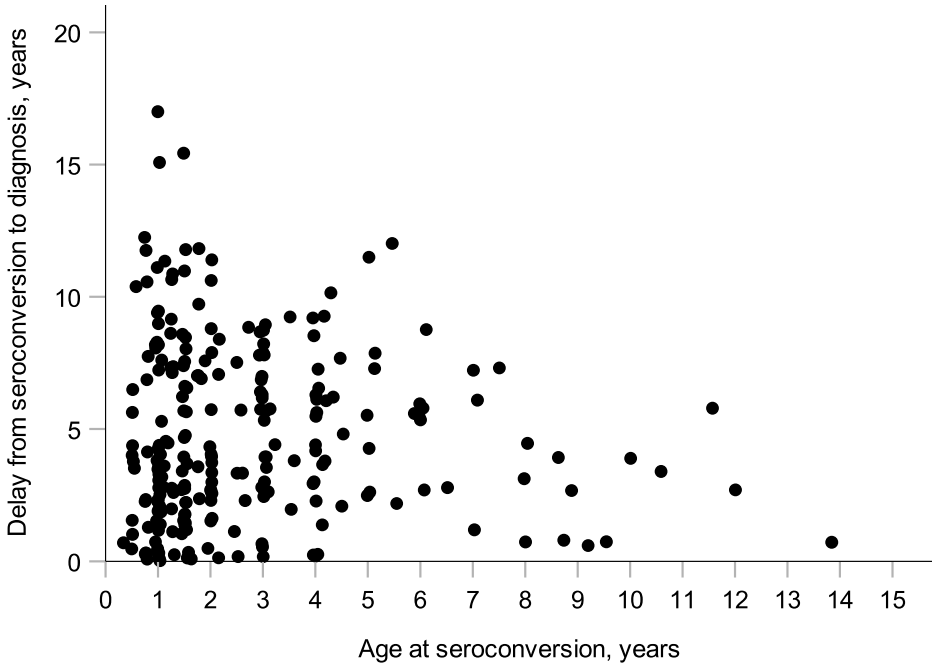

Figure 5. Delay from seroconversion to diagnosis in relation to age at seroconversion in progressors with confirmed seroconversion (N=247). Reproduced with permission from *J Clin Endocrinol Metab*.

Characterization of rapid progression to type 1 diabetes

Identifying the rapid progressors

Of the 7410 children in Study II, altogether 1550 (20.9%) tested positive for at least one autoantibody in at least two samples by 31 December 2015. The median age at seroconversion was 5.0 (range 0.2–15.1) years in the study population, and the children were followed for a median time of 16.2 (range 0.9–21.1) years from birth (Table 4). Among the seroconverted children, 248 children (16.0%) developed clinical type 1 diabetes and among the progressors 42 children (16.9%) progressed to clinical diabetes within 1.5 years after initial seroconversion. These children were considered as rapid progressors, and the rest of the progressors (*n*=206, 83.1%) were defined as the group of slower progressors. The median duration of prediabetes was 0.51 (range 0.02–1.45) years among the rapid progressors and 5.37 (range 1.53–17.0) years among the slower progressors.

Comparison of rapid progressors to slower progressors

Between rapid and slower progressors, no significant differences emerged in the distributions of the high-risk *HLA-DQB1*02/*03:02* genotype, sex, or age at seroconversion (Table 15). At seroconversion, the rapid progressors tested more often positive for multiple autoantibodies and presented with higher titers of ICA, IAA, and IA-2A than the slower progressors. However, there were no differences in positivity for any of the four autoantibodies between the groups at seroconversion, although IAA positivity tended to be more frequent among rapid progressors.

Table 15. Clinical characteristics of rapid and slower progressors.

Characteristic	Rapid progressors (*n*=42)	Slower progressors (*n*=206)	*P*-value
	N (%)		
High-risk *HLA-DQB1*02/*03:02* genotype	21 (50)	82 (40)	NS
Sex (boys)	25 (60)	117 (57)	NS
Autoantibody positivity at seroconversion			
ICA	26 (62)	116 (56)	NS
IAA	34 (81)	136 (66)	NS
GADA	24 (57)	93 (45)	NS
IA-2A	11 (26)	43 (21)	NS
Multiple autoantibodies	30 (71)	113 (55)	*
Autoantibody seroconversion <1 or ≥7 years of age	17 (41)	37 (18)	**
Distributions of non-HLA SNPs			
FUT2 major allele G homozygosity[a]	13 (68)	19 (28)	0.03[c]
PTPN22 minor allele A[b]	19 (46)	76 (38)	NS
	Median (range)		
Autoantibody titer at seroconversion			
ICA, JDFU	34.5 (4–320)	10 (3–668)	**
IAA, RU	10.5 (4.3–66.2)	8.8 (3.5–81.0)	*
GADA, RU	32.1 (5.6–310.4)	28.0 (5.8–189.5)	NS
IA-2A, RU	52.5 (2.6–108.6)	16.4 (0.4–121.0)	*
Age at seroconversion, years	1.5 (0.3–13.8)	1.9 (0.3–12.0)	NS
Age at diagnosis, years	2.4 (0.9–14.6)	8.4 (2.1–18.0)	NA
Delay from seroconversion to diagnosis, years	0.51 (0.02–1.45)	5.37 (1.53–17.0)	NA
Follow-up time, years	2.4 (0.9–14.6)	8.4 (2.1–18.0)	NA

RU=relative units. JDFU=Juvenile Diabetes Foundation units.

[a]In children with the high-risk HLA genotype.

[b]Data on the *PTPN22* SNP genotype were available for 41 rapid progressors and 200 slower progressors.

[c]Bonferroni-corrected *P*-value.

**P*<0.05

***P*<0.01

****P*<0.001

NS=non-significant.

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Comparison of rapid progressors to autoantibody-positive non-progressors

Among the 1550 autoantibody-positive children in Study II, 1302 (84.0%) remained disease-free by the end of December 2015. Compared with these non-progressors, the rapid progressors carried more frequently the high-risk HLA genotype and developed autoantibodies at a younger age, but there was no significant difference in the sex distribution between the groups (Table 16). At seroconversion, the rapid progressors tested more often positive for IAA, GADA, IA-2A, and multiple autoantibodies and demonstrated higher titers of all four autoantibodies (Table 16). The rapid progressors presented also more often with multiple biochemical autoantibodies at seroconversion (48% vs. 2%; $P<0.001$). However, the frequency of ICA positivity at seroconversion was higher among autoantibody-positive non-progressors than among rapid progressors (Table 16). This was probably related to the ICA-based primary screening in the DIPP study. The sensitivities, specificities, and predictive values for predicting rapid progression of characteristics present at seroconversion are shown in Table 17.

Table 16. Clinical characteristics of rapid progressors and autoantibody-positive non-progressors.

Characteristic	Rapid progressors (*n*=42)	AAB-positive non-progressors (*n*=1302)	*P*-value
	N (%)		
High-risk *HLA-DQB1*02/*03:02* genotype	21 (50)	319 (25)	***
Sex (boys)	25 (60)	685 (53)	NS
Autoantibody positivity at seroconversion			
ICA	26 (62)	1082 (83)	***
IAA	34 (81)	134 (10)	***
GADA	24 (57)	147 (11)	***
IA-2A	11 (26)	30 (2)	***
Multiple autoantibodies	30 (71)	70 (5)	***
Distributions of non-HLA SNPs			
FUT2 major allele G homozygosity[a]	13 (68)	23 (31)	NS[c]
PTPN22 minor allele A[b]	19 (46)	167 (24)	*[c]
	Median (range)		
Autoantibody titer at seroconversion			
ICA, JDFU	34.5 (4–320)	4 (3–512)	***
IAA, RU	10.5 (4.3–66.2)	6.9 (3.5–148.7)	***
GADA, RU	32.1 (5.6–310.4)	13.5 (5.4–342.1)	*
IA-2A, RU	52.5 (2.6–108.6)	2.3 (0.5–88.9)	***
Age at seroconversion, years	1.5 (0.3–13.8)	6.0 (0.2–15.1)	***
Follow-up time, years	2.4 (0.9–14.6)	16.5 (12.4–21.1)	NA

[a]In children with the high-risk HLA genotype.
[b]Data on the *PTPN22* SNP genotype were available for 41 rapid progressors and 696 AAB-positive non-progressors.
[c]Bonferroni-corrected *P*-value.
RU=relative units. JDFU=Juvenile Diabetes Foundation units. NA=not available. AAB=autoantibody.
**P*<0.05, ***P*<0.01, ****P*<0.001, NS=non-significant.
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Table 17. Sensitivities, specificities, positive (PPV) and negative predictive values (NPV), and odds ratios (ORs) for predicting rapid progression to type 1 diabetes of characteristics present at initial seroconversion (N=1550). Among the diabetes-associated autoantibodies at seroconversion, IAA had the highest sensitivity and IA-2A the highest specificity for predicting rapid progression. The 95% confidence intervals (CIs) are given in parentheses. The highest values in each column have been bolded.

Characteristic at seroconversion	Sensitivity, %	Specificity, %	PPV, %	NPV, %	OR (95% CI)	*P*-value
ICA positivity	62 (46–76)	21 (20–21)	2 (2–3)	95 (93–97)	0.42 (0.22, 0.79)	0.006
IAA positivity	81 (66–91)	82 (82–82)	11 (9–13)	**99** (99–100)	19.46 (8.91, 42.50)	<0.001
GADA positivity	57 (41–72)	84 (84–85)	9 (7–11)	**99** (98–99)	7.04 (3.77, 13.18)	<0.001
IA-2A positivity	26 (15–41)	95 (95–96)	13 (7–21)	98 (98–98)	6.97 (3.37, 14.42)	<0.001
Multiple (≥2) autoantibodies	71 (56–84)	88 (87–88)	14 (11–17)	**99** (99–100)	18.10 (9.11, 35.98)	<0.001
Multiple (≥2) biochemical autoantibodies	48 (33–63)	94 (93–94)	17 (12–24)	**99** (98–99)	13.22 (6.98, 25.07)	<0.001
High-risk HLA genotype	50 (35–65)	73 (73–74)	5 (3–7)	98 (98–99)	2.76 (1.49, 5.11)	<0.001
Sex (boys)	41 (26–57)	53 (53–54)	2 (2–3)	97 (96–98)	0.77 (0.41, 1.44)	0.417
FUT2 major allele G homozygosity[a]	68 (45–86)	71 (68–73)	24 (16–30)	94 (90–98)	5.26 (1.88, 14.77)	0.001
Age at seroconversion <1 year	41 (27–54)	82 (79–85)	**32** (21–42)	87 (84–90)	3.11 (1.53, 6.33)	0.001
Age at seroconversion <5 years	**86** (71–94)	52 (51–52)	5 (4–5)	**99** (98–100)	6.36 (2.66, 15.18)	<0.001
Age at seroconversion ≥7 years	14 (6–29)	64 (64–65)	1 (1–2)	96 (96–97)	0.30 (0.13, 0.72)	0.004
ICA titer ≥10 JDFU at seroconversion[b]	77 (56–90)	89 (87–88)	12 (9–14)	**99** (99–100)	23.83 (9.42, 60.31)	<0.001
ICA titer ≥20 JDFU at seroconversion[b]	65 (45–82)	**96** (95–96)	25 (17–31)	**99** (99–100)	**41.63** (17.71, 97.83)	<0.001
IAA level ≥10 RU at seroconversion[b]	53 (36–69)	65 (63–67)	16 (11–21)	92 (89–95)	2.11 (1.03, 4.32)	0.039
IAA level ≥20 RU at seroconversion[b]	27 (14–43)	87 (85–89)	20 (11–32)	90 (89–92)	2.34 (1.01, 5.41)	0.042
IAA level ≥30 RU at seroconversion[b]	24 (12–38)	93 (92–95)	31 (16–49)	91 (89–92)	4.31 (1.71, 10.87)	0.001
IA-2A level ≥50 RU at seroconversion[b]	55 (26–81)	78 (74–82)	27 (13–40)	92 (87–97)	4.28 (1.15, 15.84)	0.022

[a]Among participants with the high-risk HLA genotype.

[b]Among participants positive for the specific autoantibody reactivity.

RU=relative units. JDFU=Juvenile Diabetes Foundation units.

Double-peak distribution of age at seroconversion among rapid progressors

Rapid progression to type 1 diabetes occurred in two age peaks (Figure 6). The first peak emerged already in early childhood before the age of 5 years and the second closer to puberty at the age of 8–14 years. Most rapid progressors (n=36) belonged to the younger age group, but six children did not develop clinical diabetes until early puberty. No seroconversions between 5 and 7 years of age resulted in rapid disease progression. The double-peak distribution was exclusive for rapid progressors, not being seen for slower progressors. Compared with slower progressors, the rapid progressors became autoantibody-positive significantly more often before the age of one year or after the age of 7 years (Table 15).

Although the number of children in both subgroups of rapid progressors was modest, limiting the power of any statistical analyses, a comparison between the two subgroups revealed considerable differences in the autoantibody profiles among the rapid progressors at seroconversion. The young rapid progressors demonstrated a higher frequency of IAA positivity and higher titers of IAA than the older rapid progressors, whereas the older rapid progressors tested more often positive for GADA and had higher titers of GADA at seroconversion than the younger rapid progressors (Table 18). Although a higher number of the rapid progressors in the older subgroup turned out to be boys, the sex distribution remained non-significant. There were no statistically significant differences between the two subpopulations in the distribution of the high-risk HLA genotype, or the frequency of positivity for ICA, IA-2A, or multiple autoantibodies at initial seroconversion. Neither were the differences in the autoantibody profiles explained by the non-HLA SNPs analyzed in Study II (data not shown, except for the *FUT2* (1,2-α-Fucosyltransferase) SNP in Table 18).

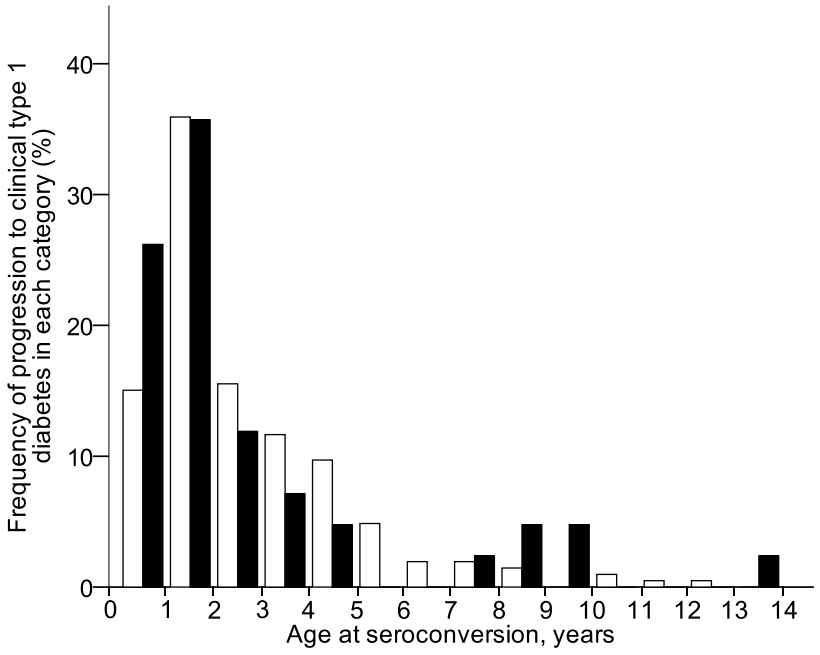

Figure 6. Seroconversion age among rapid and slower progressors. Black bars, rapid progressors. White bars, slower progressors. Reproduced with permission from *Diabetologia*.

Table 18. Clinical characteristics of young and older rapid progressors.

	Rapid progressors		
Characteristic	Young[a] (*n*=36)	Older[b] (*n*=6)	*P*-value
	N (%)		
High-risk *HLA-DQB1*02/*03:02* genotype	19 (53)	2 (33)	NS
Sex (boys)	20 (56)	5 (83)	NS
Autoantibody positivity at seroconversion			
ICA	21 (58)	5 (83)	NS
IAA	32 (89)	2 (33)	**
GADA	18 (50)	6 (100)	*
IA-2A	9 (25)	2 (33)	NS
Multiple autoantibodies	25 (69)	5 (83)	NS
FUT2 major allele G homozygosity[c]	11 (65)	2 (100)	NS
	Median (range)		
Autoantibody titer at seroconversion			
ICA, JDFU	5 (0–320)	49 (0–85)	NS
IAA, RU	9.5 (4.3–66.2)	1.4 (0.1–13.0)	**
GADA, RU	5.1 (0.1–310.4)	97.7 (5.6–158.5)	**
IA-2A, RU	0.11 (0.05–108.6)	0.12 (0.05–52.51)	NS
Age at seroconversion, years	1.4 (0.3–4.1)	9.0 (7.0–13.1)	NA
Age at diagnosis, years	2.1 (0.9–5.5)	9.7 (8.2–14.6)	NA
Delay from seroconversion to diagnosis, years	0.41 (0.02–1.45)	0.73 (0.59–1.19)	NA

RU=relative units. JDFU=Juvenile Diabetes Foundation units.
[a]Early childhood (<5 years old).
[b]Prepuberty and puberty (>7 years old).
[c]In children with the high-risk HLA genotype.
**P*<0.05
***P*<0.01
****P*<0.001
NS=non-significant.
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Non-HLA SNPs and rapid progression to type 1 diabetes

In Study II, we examined the potential associations between rapid disease progression and 25 non-HLA SNPs predisposing to type 1 diabetes (Table 5). The distributions of the SNP genotypes were compared between the groups of rapid progressors, slower progressors, and autoantibody-positive non-progressors. Although several differences in the distributions were found between the groups, after Bonferroni correction for multiple testing the only differences that remained significant were seen among children carrying the high-risk *HLA-DQB1*02/*03:02* genotype. Among such children, the rapid progressors carried more frequently the homozygous GG genotype in the *FUT2* gene than the slower progressors (68% vs. 28%; OR 5.70 [95% CI 1.89, 17.17], corrected *P*-value, P_c=0.03). In addition, the rapid progressors tended to be more often homozygous for the *FUT2* G allele than the autoantibody-positive non-progressors (68% vs. 31%; OR 4.90 [95% CI 1.66, 14.49], *P*=0.002, P_c=0.06).

Among progressors to type 1 diabetes, the delay from seroconversion to diagnosis was shorter among those homozygous for the *FUT2* gene major allele G than among those who carried the AG or AA genotype (median 3.0 vs. 4.7 years; *P*=0.03). Furthermore, among children who carried the high-risk HLA genotype and tested autoantibody-positive before the age of 6 years, a higher proportion of those with the *FUT2* GG genotype had progressed to clinical diabetes within 1.5 years after seroconversion than those carrying the *FUT2* AG or AA genotype (34% vs. 8%; *P*=0.02).

Although the intention with the analysis of the non-HLA SNPs was to assess associations with the progression rate, we observed additionally that the autoantibody-positive non-progressors carried less frequently the minor allele A in the predisposing *PTPN22* gene than either rapid progressors (46% vs. 24%; OR 2.74 [95% CI 1.45, 5.19], P_c=0.03) or slower progressors (38% vs. 24%; OR 1.93 [95% CI 1.38, 2.70], P_c=0.002), indicating a role for the *PTPN22* SNP in overall progression to type 1 diabetes.

Predictive characteristics of rapid progression in a multivariate analysis

The predictive characteristics of rapid disease progression were studied by using the Cox proportional hazards models. The variables included in the analyses comprised sex, the high-risk HLA genotype, the presence of affected FDRs at birth of study participants, age at seroconversion, multipositivity at seroconversion, and positivity and titers for ICA, IAA, GADA, and IA-2A at seroconversion. Variables that predicted rapid progression to type 1 diabetes within 1.5 years after seroconversion were the high-risk HLA genotype, age at seroconversion, multipositivity at seroconversion, IAA positivity at seroconversion, and ICA titers at seroconversion (Table 19A). Having an FDR with type 1 diabetes did not affect the risk of rapid progression.

Statistically significant variables found in the first model were included in a subsequent analysis. In the second step, age at seroconversion and titers of ICA were included as categorical variables. In the multivariate analysis, the variables identified as independent predictors of rapid progression at seroconversion were the high-risk HLA genotype, young age, positivity for multiple autoantibodies, IAA positivity, and ICA titer of >10 JDFU (Table 19B). In contrast, ICA positivity at seroconversion was inversely associated with the risk of rapid disease progression in both analyses, which might be explained by the addition of biochemical autoantibodies to the models.

Table 19. Predictive characteristics of rapid progression to type 1 diabetes in Cox proportional hazards models. The following variables were studied in the univariate analyses (A) and the multivariate analysis (B): sex, HLA-conferred disease risk, the presence of affected FDRs at birth of study participants, age at seroconversion, positivity for multiple autoantibodies at seroconversion, and positivity and titers of ICA, IAA, GADA, and IA-2A at seroconversion. The *FUT2* and *PTPN22* SNPs were not included in the models due to missing values.

A

Variable	HR (95% CI)	*P*-value
*HLA-DQB1*02/*03:02* genotype	1.64 (1.27, 2.12)	***
Sex (boys)	1.04 (0.81, 1.35)	NS
Affected FDRs	1.24 (0.82, 1.87)	NS
Age at seroconversion	0.82 (0.77, 0.87)	***
Multiple (≥2) autoantibodies at seroconversion	4.32 (2.68, 6.99)	***
ICA positivity at seroconversion	0.50 (0.35, 0.71)	***
IAA positivity at seroconversion	2.05 (1.35, 3.11)	***
GADA positivity at seroconversion	1.27 (0.84, 1.92)	NS
IA-2A positivity at seroconversion	0.96 (0.61, 1.50)	NS
ICA titers at seroconversion, JDFU	1.003 (1.001, 1.005)	**
IAA level at seroconversion, RU	1.004 (0.995, 1.014)	NS
GADA level at seroconversion, RU	1.000 (0.996, 1.003)	NS
IA-2A level at seroconversion, RU	1.007 (0.999, 1.015)	NS

B

Variable	HR (95% CI)	*P*-value
*HLA-DQB1*02/*03:02* genotype	1.58 (1.23, 2.04)	***
Age at seroconversion		***
0–4 years	5.29 (2.15, 13.05)	
5–9 years	2.33 (0.89, 6.07)	
≥10 years	1.0	
Multiple (≥2) autoantibodies at seroconversion	4.70 (3.34, 6.61)	***
ICA positivity at seroconversion	0.21 (0.12, 0.38)	***
ICA titers at seroconversion		***
≤4 JDFU	1.0	
5–10 JDFU	1.77 (0.99, 3.16)	
>10 JDFU	3.79 (2.08, 6.90)	
IAA positivity at seroconversion	1.85 (1.31, 2.61)	***

HR=hazard ratio. CI=confidence interval. JDFU=Juvenile Diabetes Foundation units. RU=relative units.

**P*<0.05

***P*<0.01

****P*<0.001

NS=non-significant.

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Characterization of slow progression to type 1 diabetes

Identifying the slow progressors

In Study III, 1528 (20.6%) of the 7410 HLA-susceptible children tested autoantibody-positive for at least one autoantibody in at least two consecutive samples by the end of 2015. Among them, 247 children (16.2%) progressed to type 1 diabetes and had confirmed seroconversion before the diagnosis. One progressor was excluded from the autoantibody analyses in Study III based on only one autoantibody-positive sample at the age of 3 months. No cord blood sample was available for this child, and therefore, it could not be determined whether the autoantibodies were derived from *de novo* synthesis.

Among the 247 progressors, 62 children (25.1%) were slow progressors, in whom type 1 diabetes was diagnosed at the earliest 7.26 years after the initial seroconversion. In contrast, there were 185 children (74.9%) with a delay from seroconversion to diagnosis of less than 7.26 years, and these children were defined as the group of other progressors. The median time from seroconversion to diagnosis was 8.7 (range 7.3–17.0) years in slow progressors and 3.0 (range 0.02–7.2) years in other progressors. In the whole study population, the median duration of the preclinical period was 4.0 (range 0.02–17.0) years (Table 4).

Comparison of slow progressors to other progressors

Compared with other progressors, the slow progressors tested less frequently positive for IA-2A and multiple autoantibodies at seroconversion, although no significant difference emerged between the groups in the frequency of positivity for multiple biochemical autoantibodies at seroconversion (Table 20). The titers of ICA and IAA at seroconversion were significantly lower among slow progressors than other progressors. In addition, the slow progressors progressed more slowly from single to multiple autoantibody positivity after seroconversion for a single autoantibody than the other progressors.

Between the two groups of progressors, there were no statistically significant differences in the distributions of the high-risk HLA genotype, sex, or the presence of FDRs with type 1 diabetes at birth of study children. No differences were found between the groups in the frequency of positivity for ICA, IAA, or GADA, or the titers of GADA and IA-2A at seroconversion (Table 20). Similar proportions of slow progressors and other progressors developed multipositivity by the time of diagnosis.

Interestingly, there was significant variation in the season of birth between slow progressors and other progressors. Slow progressors showed higher frequency of being born in the fall (from September to November) than the other progressors (31% vs. 22%), whereas the other progressors tended to be born more often in the spring (from March to May; 31% vs. 15%; *P*=0.04) (Figure 7). No similar trend was observed among non-progressors who had developed multiple autoantibodies during the follow-up.

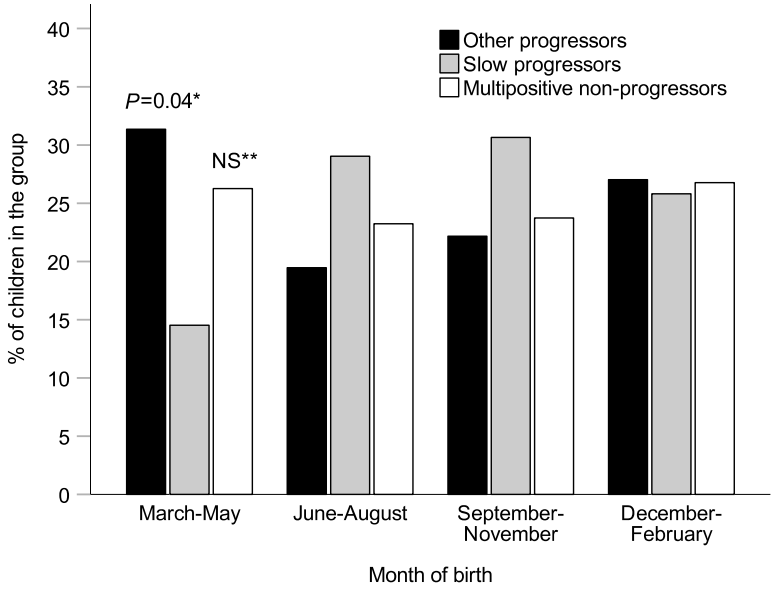

Figure 7. Season of birth among the groups of slow progressors (n=62), other progressors (n=185), and multipositive non-progressors (n=198).
*Slow progressors vs. other progressors.
**Slow progressors vs. multipositive non-progressors, other progressors vs. multipositive non-progressors.
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Table 20. Clinical characteristics of slow progressors and other progressors.

Clinical characteristic	Slow progressors (*n*=62)	Other progressors (*n*=185)	*P*-value
	N (%)		
High-risk *HLA-DQB1*02/*03:02* genotype	24 (39)	78 (42)	NS
Sex (boys)	35 (57)	107 (58)	NS
Affected FDRs	4 (7)	24 (13)	NS
Autoantibody positivity at seroconversion			
ICA	30 (48)	112 (61)	NS
IAA	44 (71)	126 (68)	NS
GADA	25 (40)	91 (49)	NS
IA-2A	5 (8)	50 (27)	**
IAA without GADA	28 (45)	74 (40)	NS
GADA without IAA	9 (15)	39 (21)	NS
IAA and GADA	16 (26)	52 (28)	NS
Multiple autoantibodies	27 (44)	117 (63)	**
Multiple biochemical autoantibodies	17 (27)	74 (40)	NS
Multipositivity during follow-up	62 (100)	182 (98)	NS
	Median (range)		
Autoantibody titers at seroconversion			
ICA, JDFU	7 (4–131)	15 (3–668)	**
IAA, RU	7.1 (3.5–40.3)	10.2 (3.6–81.0)	**
GADA, RU	21.0 (5.9–135.3)	32.9 (5.6–310.4)	NS
IA-2A, RU	9.8 (0.4–57.3)	22.9 (0.6–121.0)	NS
Age at seroconversion, years	1.5 (0.6–7.5)	2.0 (0.3–13.8)	NS
Age at diagnosis, years	11.4 (8.6–18.0)	5.5 (0.9–17.4)	NA
Delay from seroconversion to diagnosis, years	8.7 (7.3–17.0)	3.0 (0.02–7.2)	NA
Time from single to multiple autoantibody positivity, years	1.5 (0.2–8.2)	0.5 (0.2–6.0)	*
Follow-up time from seroconversion, years	8.7 (7.3–17.0)	3.0 (0.02–7.2)	NA

RU=relative units. JDFU=Juvenile Diabetes Foundation units.
**P*<0.05
***P*<0.01
****P*<0.001
NS=non-significant.
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Identifying multipositive non-progressors

Because more than 70% of multipositive children progress to clinical diabetes in 10 years, we hypothesized that the current multipositive non-progressors might eventually progress to type 1 diabetes, but at a remarkably slow pace (149). Accordingly, comparisons of phenotypically diverse slow progressors and multipositive non-progressors might reveal mechanisms counteracting the disease progression.

The group of multipositive non-progressors in Study III comprised 198 individuals who had developed multiple autoantibodies, but remained disease-free during the follow-up period until the end of 2015 and had been followed up for at least a period of 7.26 years from seroconversion. The median follow-up time among the multipositive non-progressors was 12.8 (range 7.4–20.1) years.

Comparison of slow progressors to multiple autoantibody-positive non-progressors

As expected, there were multiple similarities between the groups, but the two groups also differed in several aspects. At seroconversion, the slow progressors were significantly younger than the multipositive non-progressors (median age 1.5 vs. 3.5 years; $P<0.001$) and demonstrated higher titers of ICA (median 7 vs. 5 JDFU; $P=0.003$). The slow progressors tested more often positive for IAA (71% vs. 34%; $P<0.001$) and multiple autoantibodies at seroconversion, with ICA (44% vs. 26%; $P=0.008$) or without ICA in the analysis (27% vs. 11%; $P=0.002$).

However, the two groups shared similar distributions of the high-risk HLA genotype (39% vs. 29%; $P=0.14$), sex (boys 57% vs. 62%; $P=0.43$), and affected FDRs at birth (7% vs. 7%; $P=1.00$). At seroconversion, they demonstrated similar frequencies of positivity for ICA (48% vs. 56%; $P=0.32$), GADA (40% vs. 36%; $P=0.53$), and IA-2A (8% vs. 9%; $P=0.80$). Furthermore, the titers of IAA (median 7.1 vs. 7.0 RU; $P=0.78$), GADA (21.0 vs. 16.9 RU; $P=0.52$), and IA-2A (9.8 vs. 2.7 RU; $P=0.88$) at seroconversion did not differ between groups.

Notably, the multipositive non-progressors demonstrated higher frequency of GADA positivity at seroconversion without simultaneous IAA positivity than the slow progressors (28% vs. 15%; $P=0.03$). In contrast, the slow progressors tested more often positive for IAA at seroconversion without simultaneous GADA positivity than the multipositive non-progressors (45% vs. 26%; $P=0.005$).

Autoantibody status at seroconversion

In Study III, the majority of progressors (41.3%), including both slow (45.2%) and other progressors (40.0%), tested IAA-positive but GADA-negative at seroconversion ($P=0.48$ between progressors). However, this was true in a significantly smaller proportion of multipositive non-progressors (26%; $P=0.005$ compared with slow progressors, and $P=0.004$ compared with other progressors).

In contrast, nearly a third of the multipositive non-progressors (27.8%) tested GADA-positive at seroconversion without simultaneous IAA positivity, while the corresponding proportions among the slow and other progressors were only 14.5% and 21.1%, respectively ($P=0.04$ compared with all progressors; $P=0.03$ compared with slow progressors; and $P=0.13$ compared with other progressors). Compared with other progressors, this difference turned out to be statistically non-significant, which was probably related to the high proportion of multipositive individuals among the other progressors.

In seroconverted non-progressors with only one autoantibody during follow-up, this autoantibody was predominantly ICA (79%). Both slow (43.5%) and other progressors (63.2%) tested multipositive at seroconversion more often than seroconverted non-progressors (5.5%; $P<0.001$). The autoantibody combinations at seroconversion are shown in Table 21.

Table 21. Autoantibody combinations at seroconversion in the groups of slow progressors (n=62), other progressors (n=185), multipositive non-progressors (n=198), and single autoantibody-positive non-progressors (n=1039).

AAB combination	Slow progressors	Other progressors	Multipositive non-progressors*	Single AAB-positive non-progressors
	N (%)			
Only ICA	8 (12.9)	13 (7.0)	67 (33.8)	933 (89.8)
Only IAA	20 (32.3)	38 (20.5)	38 (19.2)	53 (5.1)
Only GADA	7 (11.3)	15 (8.1)	38 (19.2)	45 (4.3)
Only IA-2A	0 (0)	2 (1.1)	4 (2.0)	8 (0.8)
ICA + IAA	8 (12.9)	21 (11.4)	11 (5.6)	NA
ICA + GADA	1 (1.6)	17 (9.2)	14 (7.1)	NA
ICA + IA-2A	1 (1.6)	5 (2.7)	4 (2.0)	NA
IAA + GADA	5 (8.1)	14 (7.6)	5 (2.5)	NA
IAA + IA-2A	0 (0)	4 (2.2)	2 (1.0)	NA
GADA + IA-2A	0 (0)	0 (0)	1 (0.5)	NA
ICA + IAA + GADA	8 (12.9)	17 (9.2)	7 (3.5)	NA
ICA + IAA + IA-2A	0 (0)	11 (5.9)	1 (0.5)	NA
ICA + GADA + IA-2A	1 (1.6)	7 (3.8)	2 (1.0)	NA
IAA + GADA + IA-2A	0 (0)	0 (0)	0 (0)	NA
All four antibodies	3 (4.8)	21 (11.4)	4 (2.0)	NA

AAB=autoantibody. NA=not available. Reproduced with permission from *J Clin Endocrinol Metab*.
*Follow-up time ≥7.26 years from seroconversion to autoantibody positivity.

Non-HLA SNPs and slow progression to type 1 diabetes

We analyzed the potential associations between slow disease progression and the previously reported non-HLA SNPs predisposing to type 1 diabetes (Table 5). Multiple SNPs were associated with the delay from seroconversion to diagnosis (Table 22), but after corrections for multiple comparisons these associations did not reach statistical significance. Also, after corrections for multiple testing, no significant differences were observed in the non-HLA SNP distributions between the groups of slow progressors, other progressors, and multipositive non-progressors (Table 23).

Not surprisingly, the predisposing SNPs in the *PTPN22*, *INS* (rs689), and *PHTF1* genes were associated with overall progression to type 1 diabetes and survived the corrections for multiple testing (Table 24). Also, the CC, CT genotype of the predisposing *GPR183/EBI2* SNP tended to associate with overall progression to type 1 diabetes in the whole study population independently of seroconversion status (progressors 51% vs. non-progressors 41%; P=0.008, P_c=0.06).

Table 22. Predisposing SNPs associated with the delay from seroconversion to diagnosis of type 1 diabetes in progressors. NS=non-significant, P_c=corrected *P*-value by using the Benjamini-Hochberg step-up procedure to control the false discovery rate.

Gene	SNP	Genotype (n)	Median (range)	*P*-value	P_c
RGS1	rs2816316	TT (148)	4.6 (0.02–17.0)	0.03	NS
		GT, GG (55)	3.5 (0.12–11.8)		
FUT2	rs601338	AA, AG (133)	4.8 (0.1–17.0)	0.04	NS
		GG (69)	3.1 (0.02–12.3)		
LOC646538	rs630115	GG (108)	4.6 (0.1–15.1)	0.01	NS
		AA, AG (118)	3.4 (0.02–17.0)		
IGF2BP2	rs4402960	GT, GG (219)	4.3 (0.02–17.0)	0.04	NS
		TT (18)	2.8 (0.2–7.6)		
High-risk HLA genotype					
SH2B3	rs3184504	TT (12)	2.4 (0.1–11.0)	0.04	NS
		CT, CC (75)	4.1 (0.1–12.0)		
CTSH	rs3825932	TT (16)	2.3 (0.2–8.0)	0.01	NS
		CT, CC (81)	4.1 (0.1–12.0)		
SLC30A8	rs13266634	TT (8)	7.5 (0.3–10.6)	0.04	NS
		CT, CC (91)	3.5 (0.1–12.0)		
FUT2	rs601338	AA, AG (56)	4.7 (0.3–12.0)	0.02	NS
		GG (31)	2.7 (0.1–11.4)		
Moderate-risk HLA genotype					
ERBB3	rs2292239	AA (15)	2.4 (0.1–15.4)	0.007	NS
		AC, CC (120)	4.7 (0.02–17.0)		
IL2RA	rs11594656	TT, AT (112)	4.4 (0.1–17.0)	0.04	NS
		AA (3)	1.2 (0.2–3.4)		
GIMAP5	rs6965571	GG (100)	5.1 (0.1–15.4)	0.03	NS
		AG, AA (36)	2.6 (0.02–17.0)		
SLC30A8	rs13266634	CC, TT (76)	3.9 (0.1–15.4)	0.03	NS
		CT (63)	5.7 (0.02–17.0)		
Boys					
LOC646538	rs630115	GG (61)	5.6 (0.3–15.1)	0.004	NS
		AG, AA (68)	3.3 (0.1–17.0)		
IGF2BP2	rs4402960	GT, GG (126)	4.5 (0.1–17.0)	0.02	NS
		TT (8)	2.7 (0.2–4.1)		
NRP1	rs2666236	AA, AG (76)	5.3 (0.1–17.0)	0.04	NS
		GG (40)	3.1 (0.1–12.3)		
Girls					
KIAA0350-CLEC16A	rs2903692	GG (35)	3.0 (0.1–10.6)	0.045	NS
		AG, AA (49)	5.3 (0.02–11.4)		
AFF3	rs9653442	CC (16)	2.3 (0.1–8.0)	0.02	NS
		CT, TT (71)	4.4 (0.02–11.8)		
INS	rs689	TT (2) 9.3	9.3 (8.0–10.7)	0.04	NS
		AA, AT (101)	3.6 (0.02–11.8)		

Table 23. Comparison of the distributions of the predisposing SNPs in the groups of slow progressors, other progressors, and multipositive non-progressors. P_c=corrected *P*-value by using the Benjamini-Hochberg procedure to control the false discovery rate. Reproduced with permission from *J Clin Endocrinol Metab*.

P^a = Slow progressors vs. other progressors

P^b = Slow progressors vs. multipositive non-progressors

P^c = Other progressors vs. multipositive non-progressors

Gene	SNP	Genotype	Slow progressors	Other progressors	Multipositive non-progressors	P^a	P_c^a	P^b	P_c^b	P^c	P_c^c
			N (%)	N (%)	N (%)						
DQX1	rs6546909	AA	29 (57)	108 (71)	94 (76)	0.06	0.84	**0.01**	0.81	0.38	0.88
AFF3-LOC150577	rs9653442	CC	4 (8)	35 (23)	24 (19)	**0.02**	0.84	0.07	0.81	0.46	0.89
GPR183/EBI2	rs9585056	CC, CT	28 (55)	75 (49)	46 (37)	0.49	0.98	**0.03**	0.81	**0.04**	0.58
PHTF1	rs6679677	AA, AC	18 (35)	60 (40)	33 (27)	0.53	0.98	0.25	0.81	**0.02**	0.48
IL2RA	rs11594656	TT	35 (69)	110 (74)	77 (62)	0.47	0.98	0.41	0.81	**0.04**	0.58
PTPN22	rs2476601	AA, AG	20 (33)	74 (41)	48 (27)	0.30	0.98	0.37	0.81	**0.007**	0.27
CD226	rs763361	TT, CT	43 (74)	141 (79)	104 (65)	0.46	0.98	0.18	0.81	**0.004**	0.27

Table 24. Predisposing SNPs associated with overall progression to type 1 diabetes (T1D). P_c=corrected *P*-value by using the Benjamini-Hochberg step-up procedure to control the false discovery rate. Reproduced with permission from *J Clin Endocrinol Metab*.

Gene	SNP	Predisposing genotype to T1D	Progressors	Non-progressors	*P*-value	P_c
			N (%)	N (%)		
PTPN22	rs2476601	AA, AG	97 (38.2)	483 (23.4)	<0.001	<0.001
INS	rs689	AA	199 (78.7)	1268 (63.5)	<0.001	<0.001
NRP1	rs2666236	AA, AG	150 (70.8)	738 (62.3)	0.02	0.13
CD226	rs763361	TT, CT	192 (76.8)	1232 (70.2)	0.03	0.16
PHTF1	rs6679677	AA, AC	78 (38.0)	282 (23.9)	<0.001	<0.001
PRKCQ	rs11258747	TT	20 (9.5)	65 (5.5)	0.03	0.16
GPR183 / EBI2	rs9585056	CC, CT	107 (50.5)	481 (40.7)	0.008	0.06

Approaches to screening for risk of type 1 diabetes

Role of islet cell antibodies in disease prediction

In Study I, we explored the role of ICA in preclinical screening for risk of type 1 diabetes. Sensitivity, specificity, and PPV of individual autoantibody reactivities and multipositivity for predicting type 1 diabetes are presented in Table 25. Most importantly, the disease specificity and PPV of positivity for multiple biochemical autoantibodies were significantly higher than for ICA, when the threshold for ICA positivity was set at 2.5 JDFU, but not when it was set at 10 JDFU.

Among multipositive children, there were no ICA-negative individuals and positivity for individual biochemical autoantibodies occurred considerably less often in multipositive children than positivity for ICA. Altogether 48 (68%) of the multipositive children tested positive for IAA, 52 (73%) for GADA, 40 (56%) for IA-2A, and 43 (61%) for ZnT8A. All available samples from these children had been analyzed for ICA, IAA, GADA, IA-2A, and ZnT8A. When ICA was omitted from the screening, 21 multipositive children (29.6%) would have been missed (Figure 8). Among these children, there was one progressor with multiple biochemical autoantibodies at the time of diagnosis.

Removing ICA from the autoantibody analyses did not affect the association of the high-risk HLA genotype with multipositivity compared with the moderate-risk HLA genotypes. With ICA in the analysis, 32 (35%) of the seroconverted children in the high-risk HLA group and 39 (21%) of those in the moderate-risk HLA group developed multiple autoantibodies (P=0.01) by 15 years of age. The corresponding figures without ICA in the analysis were 26 (58%) and 24 (35%; P=0.02).

Table 25. Sensitivities, specificities, and positive predictive values (PPV) of individual autoantibodies and their combinations by the age of 15.5 years when at least two consecutive positive samples were required for autoantibody positivity (A) and when one positive sample was required for positivity (B). Confidence intervals (95%) are shown in parentheses. JDFU=Juvenile Diabetes Foundation units. Table A reproduced with permission from *J Clin Endocrinol Metab*.

A

Autoantibody type	Sensitivity, %	Specificity, %	PPV, %
ICA	91 (76–98)	78 (78–78)	13 (11–14)
IAA	77 (61–89)	96 (95–96)	39 (31–45)
GADA	71 (55–84)	96 (95–96)	36 (28–43)
IA-2A	69 (53–81)	98 (98–99)	57 (44–68)
ZnT8A	71 (56–84)	98 (97–98)	52 (41–61)
Multiple (≥2) autoantibodies	91 (77–98)	96 (96–96)	45 (38–48)
Multiple (≥2) biochemical autoantibodies	89 (74–96)	98 (98–98)	62 (52–67)
ICA threshold for positivity ≥10 JDFU	89 (74–96)	96 (95–96)	43 (36–47)

B

Autoantibody type	Sensitivity, %	Specificity, %	PPV, %
ICA	91 (76–98)	77 (76–77)	13 (10–13)
IAA	80 (63–91)	91 (90–91)	24 (19–27)
GADA	74 (58–87)	93 (93–94)	29 (22–34)
IA-2A	77 (62–88)	97 (97–98)	52 (41–60)
ZnT8A	80 (64–91)	96 (95–96)	42 (33–47)
Multiple (≥2) autoantibodies	91 (77–98)	95 (94–95)	38 (32–41)
Multiple (≥2) biochemical autoantibodies	91 (78–98)	98 (97–98)	58 (49–62)

Definition of autoantibody positivity

In Study I, we examined whether the definition of autoantibody positivity as positivity for at least one autoantibody in at least one vs. two consecutive samples affected the screening for islet autoimmunity. When two consecutive positive samples instead of only one positive sample were required for autoantibody positivity, the cumulative proportion of children with only one positive autoantibody was markedly lower, while the life-table curve for children with multiple autoantibodies remained similar (Figure 8). Sensitivies, specificities, and PPV for predicting type 1 diabetes by using both definitions are shown in Table 25.

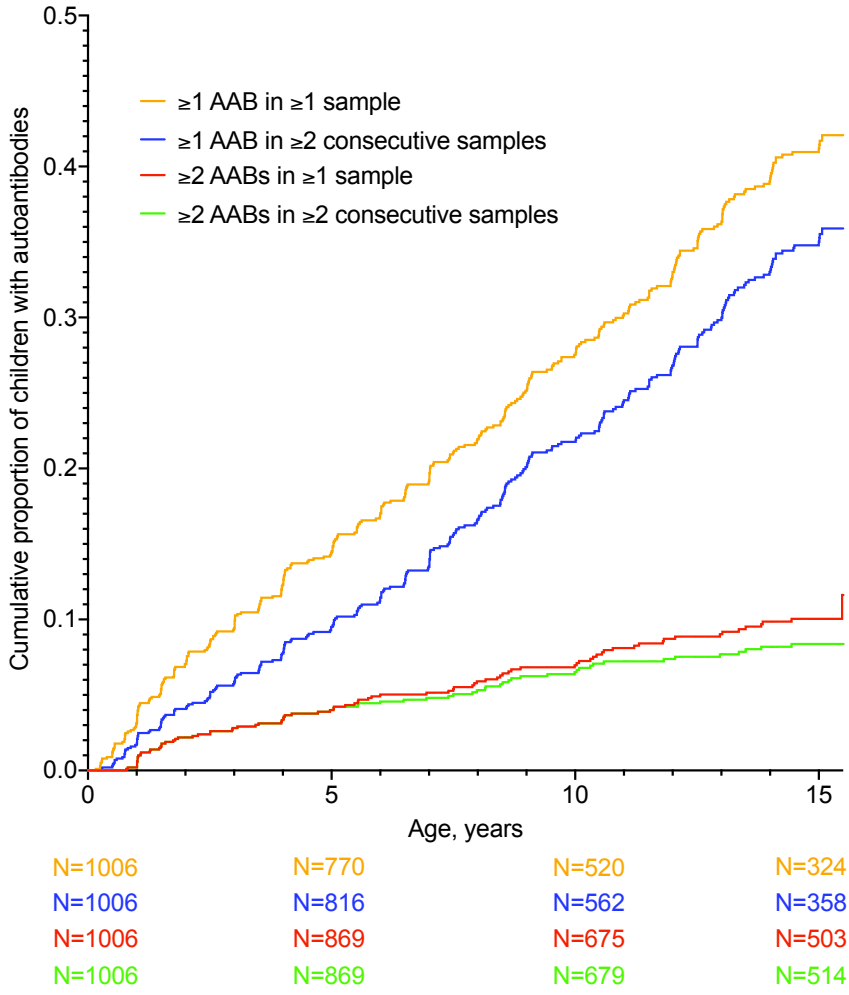

Figure 8A. Development of at least one (orange line) or at least two (red line) autoantibodies by the age of 15.5 years when autoantibody positivity was defined as positivity for ICA, IAA, GADA, IA-2A, and/or ZnT8A in at least one sample; development of at least one (blue line) or at least two (green line) autoantibodies when at least two consecutive samples were required for autoantibody positivity in 1006 children with increased HLA predisposition for type 1 diabetes.

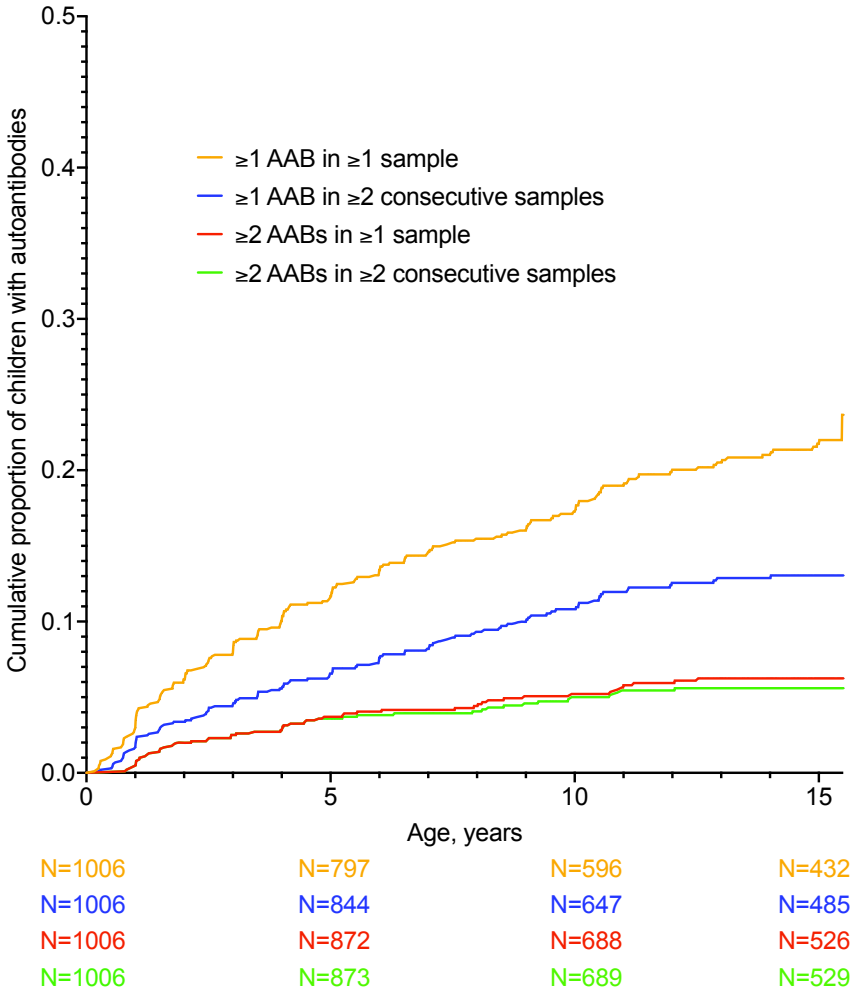

Figure 8B. Development of at least one (orange line) or at least two (red line) autoantibodies by 15.5 years of age when autoantibody positivity was defined as positivity for the biochemical autoantibodies (IAA, GADA, IA-2A, and/or ZnT8A) in at least one sample; development of at least one (blue line) or at least two (green line) autoantibodies when positivity was defined as positivity for the biochemical autoantibodies in at least two consecutive samples in 1006 children with increased HLA predisposition for type 1 diabetes.

SUMMARY OF FINDINGS

The main findings of this thesis are summarized below.

1. Among HLA-predisposed children recruited from the general population, rapid progressors to type 1 diabetes can be characterized by factors present at seroconversion to autoantibody positivity, including young age, higher autoantibody titers of especially IAA, IA-2A, and ICA, positivity for multiple autoantibodies, and higher prevalence of the high-risk *HLA-DQB1*02/*03:02* genotype and the homozygous secretor GG genotype of the *FUT2* SNP predisposing to type 1 diabetes.

2. The double-peak profile of seroconversion age among the rapid progressors demonstrates for the first time that rapid progression to type 1 diabetes may occur not only in young children below the age of 5 years, but also in children closer to puberty. At seroconversion, young rapid progressors are characterized by IAA positivity and high IAA titers, while older rapid progressors are characterized by GADA positivity and high GADA titers. This indicates age-dependent heterogeneity in the triggers of rapid progression.

3. Slow progressors to type 1 diabetes demonstrate lower titers of IAA and ICA, lower frequency of positivity for IA-2A or multiple autoantibodies at seroconversion, and a longer delay from single to multiple autoantibody positivity than other progressors.

4. Season of birth is associated with pace of disease progression from seroconversion to clinical diabetes. Disease progression is accelerated in children born in the spring and slower among children born in the fall.

5. None of the investigated SNPs predisposing to type 1 diabetes are associated with slow disease progression after correction for multiple testing. The predisposing *PTPN22*, *INS* (rs689), and *PHTF1* SNPs are associated with overall progression to clinical type 1 diabetes. The *EBI2/GPR183* SNP might also be associated with overall progression to type 1 diabetes.

6. Over the first 15 years of life, the seroconversion rate to positivity for biochemically defined autoantibodies decreases with advancing age, but the ICA seroconversion rate increases continuously towards puberty.

7. Among children with a single biochemical autoantibody at seroconversion, IAA or GADA is most often the primary biochemical autoantibody, but in young children, ZnT8A is the second most common primary autoantibody after IAA. This is the first study to demonstrate that ZnT8A may appear early in the disease process.

8. Inverse seroconversions and fluctuations of autoantibody positivity are observed most frequently for IAA, followed by ICA, especially among non-progressors. Among progressors, ICA is the most stable and persistent autoantibody. Reversion to complete autoantibody negativity occurs rarely among children with multiple biochemical autoantibodies.

9. Among progressors to type 1 diabetes, the loss of IAA positivity is associated with delayed progression from seroconversion to diagnosis.

DISCUSSION

Dynamics of islet autoantibodies in childhood

In this thesis, we explored the dynamics of islet autoantibodies in HLA-predisposed children to type 1 diabetes during prospective follow-up from birth up to 15 years of age. This extensive general population-based study was carried out in Finland, which has the highest incidence of type 1 diabetes globally. Follow-up data from the same cohort up to the age of 2 and 5 years have been previously published (7, 112).

During the first 15 years the overall seroconversion rate increased, which was mostly explained by a continuous increase in the ICA seroconversion rate with increasing age. However, the biochemical autoantibodies demonstrated decreasing seroconversion rates with advancing age. Among the islet autoantibodies, ICA became the dominating autoantibody after the age of 3 years, but IAA seroconversions prevailed in younger children. The cumulative proportion of multipositive individuals showed a steady increase up to the age of 15 years, but positivity for multiple biochemical autoantibodies developed rarely in children older than 10 years. Multipositivity within a year from seroconversion indicated progression to clinical diabetes, but this observation no longer reached statistical significance when ICA was omitted from the autoantibody analysis.

By 15 years of age, there were no differences in the overall seroconversion rate between boys and girls. This was somewhat surprising considering that type 1 diabetes is more common among males than females after puberty (298). However, the cumulative frequency of seroconversion to biochemical autoantibody positivity was slightly higher among boys than girls. Also, the majority of children diagnosed with type 1 diabetes after the age of 10 years were boys, while in younger children there was no sex difference in the proportion of progressors. This might indicate that boys are more susceptible to progression to clinical diabetes when approaching puberty.

We described for the first time systematically the role of ZnT8A in the prediction of type 1 diabetes as a part of the autoantibody repertoire used for screening purposes. During the first 15 years ZnT8A emerged commonly in young children and often as the primary autoantibody preceding even IAA. Together with IA-2A, ZnT8A demonstrated the highest specificity for type 1 diabetes, and, as the primary autoantibody at seroconversion, was strongly predictive of clinical disease. This probably reflects the strong beta-cell specificity of ZnT8.

Generally speaking, ZnT8A has been considered to appear relatively late in the disease process, but according to the current observations, this might not be entirely true. More likely, ZnT8A might reflect an aggressive disease process. Clinically, positivity for ZnT8A at diagnosis has been linked to a high risk of diabetic ketoacidosis and predicts increased need for exogenous insulin after diagnosis (163). Accordingly, our observations suggest that ZnT8A emerging early during the preclinical process indicates a high risk of type 1 diabetes. However, this conclusion should be interpreted cautiously due to the limited size of the study population.

The dynamics of islet autoantibodies might be utilized in preclinical screening when estimating the risk of type 1 diabetes in multipositive individuals. We observed that the titers of ZnT8A together with ICA and IA-2A increased in progressors months before diagnosis compared with matched multipositive controls. This is consistent with earlier studies reporting that ZnT8A is often seen together with ICA and/or IA-2A at diagnosis, but rarely with simultaneous IAA or GADA positivity

(154, 159, 163). The prevalence of ZnT8A at diagnosis in the current study (66%) was in accordance with earlier observations (159, 164).

As might be expected considering the heterogeneous nature of type 1 diabetes, we demonstrate that the primary autoantibody is characteristic of age. In young children, islet autoimmunity often begins with IAA or ZnT8A positivity, while at preschool age GADA and IA-2A-driven autoimmunity increases. After the preschool years, ICA begins to dominate as the primary autoantibody, but if remaining as a single autoantibody it does not mediate any risk for clinical diabetes. This indicates the existence of multiple paths of islet autoimmunity and highlights the concept of disease endotypes. Moreover, there are apparent differences in the primary autoantibody signatures between progressors and non-progressors at seroconversion, with IAA and ZnT8A associating most often with progression to clinical disease. The primary autoantibody might reflect the driving events of islet autoimmunity. However, assessing these mechanisms was beyond the scope of this thesis.

Notably, GADA as the first single autoantibody at seroconversion was rarely seen in progressors. Instead, GADA was the predominant primary autoantibody among multipositive non-progressors. This might be explained by the typically older age at primary GADA positivity relative to primary IAA or ZnT8A positivity (52, 151, 320). In the DIPP study, no difference in disease progression was previously observed between individuals with IAA or GADA as the first autoantibody who had also developed secondary autoantibodies (117). The substantially longer follow-up in the current study has added to the number of older children with GADA as the first autoantibody in whom the disease process has not yet advanced further.

Considering the different genetic backgrounds (117, 128, 129, 320, 340) and etiological associations of the two major endotypes of islet autoimmunity (266), the current observations support the idea of heterogeneity in the disease process and suggest that age and the stage of immunological maturation contribute substantially to the features of islet autoimmunity. Environmental exposures in children of various ages might differ considerably. As an example, the frequency of infections often increases at preschool age, as children start daycare. Early childhood infections have, in fact, been temporally associated with the onset of islet autoimmunity in several prospective studies (249–251). These findings constitute a framework for understanding the disease heterogeneity and for individualizing the approach of predicting and preventing type 1 diabetes. The increase in ICA reactivity towards puberty might reflect the fact that ICA positivity is commonly seen in adults (395).

The labile state of autoantibodies might be of value when evaluating the risk of type 1 diabetes. Among the islet autoantibodies, inverse seroconversions and fluctuations of IAA were most common, followed by ICA in children who did not develop clinical type 1 diabetes. Inverse seroconversions of IAA and ICA were inversely associated with peak IAA and ICA titers, respectively. Our findings support previous observations stating that IAA appear at a young age, but are relatively unstable in character (7, 52, 112, 115, 117, 151, 191, 320).

Since IAA appear early in the disease process, it has been considered whether insulin is the primary autoantigen in type 1 diabetes (113, 114). In this respect, it was noteworthy that the loss of IAA positivity was associated with delayed progression from seroconversion to clinical diabetes compared with persistent IAA positivity. A similar finding has been previously described in multipositive children (194). This phenomenon evokes questions about why the insulin-specific autoantibody response might be suppressed during disease progression. First, the humoral immune response might be attenuated, but the cell-mediated immune response towards insulin might persist. Second, considering the heterogeneity in the residual beta-cell function in long-term type 1 diabetes,

it is possible that the beta cells in some individuals possess the ability to escape the immune attack rather than being entirely destroyed (58, 59). In non-obese diabetic (NOD) mice, a subset of beta cells has been observed to survive immune attack supposedly due to mechanisms capable of hiding the beta cells from the immune system (99). Third, some evidence exists that B cells might be actively involved in the pathogenesis, and when the support provided by humoral immunity is removed, disease progression might be decelerated (56).

At the beginning of the DIPP study, it was predicted that approximately 7% of children carrying the high-risk *HLA-DQB1*02/*03:02* genotype and 2–3% of children with moderate-risk genotypes in the Finnish population might progress to clinical type 1 diabetes by 15 years of age (385). In this thesis, we observed this prediction to hold true. This emphasizes the role of HLA-mediated antigen presentation in the disease pathogenesis. However, the children in the current study cohort were born in 1994–1997, and due to the evolving disease process behind type 1 diabetes, the HLA associations are changing and might be different among more recently born individuals (204).

Characteristics of rapid disease progression

This is the first prospective study to demonstrate that children with rapid progression to type 1 diabetes can be characterized by demographic, genetic, and immunological factors present at seroconversion to autoantibody positivity. These include young age, high autoantibody titers of especially IAA, IA-2A, and ICA, positivity for multiple autoantibodies, and higher prevalence of the high-risk *HLA-DQB1*02/*03:02* genotype and the homozygous secretor genotype of the *FUT2* SNP predisposing to type 1 diabetes. Sex does not affect the pace of disease progression. In the multivariate models, independent predictors of rapid progression were identified as the high-risk HLA genotype, young age at seroconversion, multipositivity, IAA positivity, and high ICA titer at seroconversion. Characteristics associated with the progression rate in this thesis are summarized in Table 26.

Our findings are consistent with previous observations that multipositivity at a young age, the emergence of IA-2A as the primary biochemical autoantibody, and high initial titers of IAA strongly indicate rapid progression to clinical diabetes in childhood (117, 149). Several of the characteristics associated with overall progression to clinical diabetes were found also to be factors related to the progression rate. These include high titers of IAA and ICA at seroconversion, multipositivity at a young age, and persistent IAA positivity compared with unstable IAA (51, 107, 108, 130, 149, 175, 193).

As a novel discovery, we observed a double-peak profile of the seroconversion age among rapid progressors. The first peak occurred in young children and the second closer to puberty. Young rapid progressors were characterized by high frequency of IAA positivity and high titers of IAA at seroconversion, while the older subgroup demonstrated high frequency of GADA positivity and high titers of GADA at seroconversion. The differences in the autoantibody profiles between the subgroups were not explained by distributions of the HLA genotype or the analyzed non-HLA SNPs. The duality of seroconversion age among rapid progressors raises questions about whether the disease process is similar in all forms of rapid progression (117). The environmental triggers of islet autoimmunity might differ between young and prepubertal rapid progressors, both of whom are undergoing sensitive stages of development. The idea of multiple triggers behind rapid progression is supported by the fact that the majority of the older rapid progressors had tested autoantibody-negative within a year before seroconversion. This might suggest that the mechanisms mediating rapid progression are independent

of the triggers of islet autoimmunity. Considering the screening for risk of type 1 diabetes, the current observations indicate that in the future the assessment of islet autoantibodies might be targeted to selected age groups.

This is the first study to report an association between the *FUT2* SNP predisposing to type 1 diabetes and the pace of disease progression. Rapid progressors with the high-risk HLA genotype demonstrated an increased prevalence of the homozygous *FUT2* secretor genotype. This is, however, not the *FUT2* genotype predisposing to type 1 diabetes (220). The *FUT2* gene encodes 1,2-α-fucosyltransferase (FUT2), the enzyme responsible for synthesis of soluble ABO histo-blood group antigens found in bodily fluids and on intestinal mucosa, thereby defining the human secretor status (396). Non-secretor individuals homozygous for the non-functional *FUT2* allele A do not express the functional enzyme, and, as a result, lack the soluble ABO antigens from secretions (397). The *FUT2* secretor genotype has been reported to predispose to several intestinal pathogens, including rotavirus and norovirus, whereas the non-secretor genotype provides protection against symptomatic norovirus infection (398–401). This effect is probably mediated through the function of ABO histo-blood group antigens as intestinal receptors for many pathogens, including rotavirus and norovirus (402, 403). Notably, it has been reported that the incidence of type 1 diabetes in Australia and the United States has decreased among children under the age of 5 years after the introduction of the rotavirus vaccine into the national vaccination program, although it is controversial whether rotaviruses induce islet autoimmunity (254, 255, 404). Recent studies have also suggested an association between ABO blood type and susceptibility to COVID-19 disease (405). Intriguingly, high concentrations of α-1,2-linked fucosylated glycans in human milk mediate protection against infectious diarrhea in breastfed infants (406). The impact of the *FUT2* secretor status on the infant gut microbiota remains incompletely understood. According to the literature, the maternal *FUT2* genotype modifies the composition of the gut microbiota in cesarean-born infants, but not in vaginally born infants (407). The association of the homozygous *FUT2* secretor genotype with rapid disease progression suggests that high levels of soluble ABO antigens in bodily secretions might contribute to rapid progression to type 1 diabetes.

Characteristics of slow disease progression

Similarly, we set out to identify the demographic, genetic, and immunological characteristics of slow progressors to type 1 diabetes. The slow progressors tested less frequently positive for IA-2A and multiple autoantibodies and demonstrated lower titers of ICA and IAA at seroconversion than the other progressors. The delay from single to multiple autoantibody positivity was longer among slow progressors than other progressors. There was an interesting trend in the season of birth between progressors. The slow progressors were frequently born in the fall, while other progressors were born more often in the spring. However, no specific characteristics at seroconversion were explicitly predictive of slow progression. The main challenge was to distinguish characteristics predictive of slow progression from the ones associated with overall progression to type 1 diabetes.

The characteristics of slow progressors to type 1 diabetes have been explored in several studies. In the BABYDIAB study, slow progression from multipositivity to diagnosis was associated with delayed appearance of IA-2A (150). Certain differences were also found in the distributions of non-HLA SNPs between progressors (150). In the TEDDY study, a genetic risk score based on multiple HLA and non-HLA SNPs predicted the pace of disease progression (334). In the DAISY study, slow

Table 26. Summary of characteristics associated with the progression rate to clinical type 1 diabetes based on observations in this thesis.

Clinical characteristic	Progression to type 1 diabetes	Rapid progression	Slow progression	Possible ultraslow progression (multiple autoantibodies)	Non-progressive autoimmunity
Season of birth	Any	Spring	Not spring / Fall	Any	Any
Age at seroconversion	Young	Young or early puberty	Young	Preschool and later	After preschool
HLA risk genotype	High risk	High risk	High risk	High or moderate	Moderate risk
Autoantibody positivity at seroconversion	Multipositive IAA ZnT8A	Multipositive IA-2A	Multipositive IAA without GADA	GADA without IAA	One autoantibody Mostly ICA
Progression to multipositivity	Within a year from seroconversion	Fast	Slower than rapid progressors	Slower than progressors	Not multipositive
Autoantibody titers at seroconversion	High ICA High IAA	High ICA High IAA High IA-2A	Moderate ICA Moderate IAA	Moderate ICA Moderate IAA	Low ICA
Associated SNPs	*INS* *PTPN22* *PHTF1* *EBI2*	*FUT2* supersecretor genotype (+ high risk HLA genotype and age ≤5 years at seroconversion)	None found	NA	NA
Primary autoantibody	IAA ZnT8A	IA-2A*	ZnT8A?*	GADA	ICA
Autoantibody stability	Persistent ICA Relatively persistent IAA	Persistent IAA	Transient IAA	Fluctuating IAA Transient ICA	Fluctuating GADA Fluctuating ICA Transient ICA Transient IAA Transient IA-2A

*As previously observed by Ilonen et al. in (151).

progressors were characterized by later onset of islet autoimmunity, lower frequency of IAA positivity, and lower autoantibody titers, especially IAA, and demonstrated delayed progression to multipositivity compared with other progressors (337). Our observations are in accordance with earlier studies, implying that delayed development of multipositivity, lower frequency of IA-2A positivity at seroconversion, and lower initial autoantibody titers of especially IAA are characteristic of slow progressors. However, we found no associations between the investigated non-HLA SNPs and slow disease progression after correction for multiple testing.

Since most multipositive children progress to clinical diabetes within 15 years, a proportion of the current multipositive non-progressors may still progress to clinical diabetes, but extremely slowly (149). Comparisons of multipositive individuals with phenotypically distinguished slow progressors might promote the discovery of mechanisms capable of slowing down disease progression. In this thesis, we demonstrated a GADA-positive signature among multipositive non-progressors that was distinct at seroconversion from both progressors and single autoantibody-positive non-progressors. Apart from seroconverting to autoantibody positivity at an older age than progressors, the multipositive non-progressors tested more frequently GADA-positive at seroconversion without simultaneous positivity for IAA. In contrast, the progressors tested more often IAA-positive in the absence of GADA positivity.

Accordingly, the primary autoantibody seems to some extent predict the pace of progression to clinical diabetes. In a multicohort analysis comprising data from five prospective studies, GADA was the most frequent autoantibody in the first multipositive sample among individuals who remained disease-free for more than 10 years after seroconversion, suggesting that initial GADA positivity might indicate slower pace of the disease process (338). However, this might not hold entirely true since in this thesis high initial titers of GADA were commonly seen in prepubertal rapid progressors. This raises questions of whether e.g. GAD epitope-specific responses affect the pace of disease progression in GADA-positive individuals (135, 178).

It is also enticing to consider whether GADA might reflect the action of mechanisms opposing disease progression. The autoantigen of GADA, glutamic acid decarboxylase, is essential in the synthesis of the neurotransmitter GABA, which has been reported to exhibit anti-inflammatory properties, enhance beta-cell survival, and promote the maintenance of the human beta-cell mass (408). Intriguingly, high levels of GABA after birth indicate future seroconversion to primary IAA positivity, but do not contribute to future risk of primary GADA positivity (359). This might reflect the fact that the function of GAD in beta cells may not be affected until an older age in GADA-driven autoimmunity. However, increased levels of glutamic acid and decreased levels of metabolites derived from it precede seroconversion to GADA-first in young children (359). This might suggest that an abnormal glutamic acid metabolism is causally related to the underlying autoimmune response against GAD and/or to its altered function. Whereas the IAA-first endotype has been linked to the type 1 diabetes-associated coxsackievirus B1 infections, initial GADA positivity is likely to originate from a different etiological background (266).

None of the investigated non-HLA SNPs were associated with slow progression after corrections for multiple comparisons. This was not surprising since the HLA region contributes to approximately 50% of the genetic heritability of type 1 diabetes, whereas the non-HLA loci together account for an estimated 30% of the heritability (209). However, assessing this possibility was mechanistically important to rule out significant associations. As expected, the predisposing SNPs in the *PTPN22*, *INS* (rs689), and *PHTF1* genes were associated with overall progression to clinical

diabetes. The *PTPN22* and *PHTF1* SNPs are in almost perfect LD, meaning that individuals who carry one particular SNP allele at one site in the genome also often carry specific alleles at another nearby site. Both the *PTPN22* and *PHTF1* SNPs are located within a massive LD block containing in addition several other genes (210, 409). Therefore, the association of the *PHTF1* SNP with overall progression might be explained by LD with the *PTPN22* SNP, which strongly predisposes to type 1 diabetes.

Considering the development of immunological tolerance and the maturation of the immune system, it is noteworthy that the predisposing *EBI2*/*GPR183* SNP tended to be associated with overall progression to clinical diabetes. The gene product of *EBI2* is a chemokine receptor, the Ebstein-Barr virus-induced G protein-coupled receptor (EBI2), which guides the localization of immune cells in lymphoid tissues (410). In mice, EBI2 deficiency alters the migration rate of thymocytes into thymic medulla during negative selection and might affect the development of central tolerance (411). Deficiencies in EBI2 or its ligand 7α,25-dihydroxycholesterol result in impaired humoral immune responses in mice (410). Furthermore, efficient induction of adaptive immune responses in mice requires EBI2-mediated sensing of oxysterol gradients (412). Considering the essential role of EBI2 for the localization of B cells in lymphoid tissues, EBI2-orchestrated humoral immune responses might be involved in the pathogenesis of type 1 diabetes, which is highly characterized by autoantibody production against islet antigens. In newly diagnosed patients with type 1 diabetes, two different phenotypes of insulitis have been described in which the number of CD20-positive B cells in the insulitic lesion is either high or low, reflecting a considerably different age at diagnosis (64). Also, since the migration of immune cells is controlled by a variety of chemokine receptors, EBI2 might play a role in the localization of immune cells into tissues outside the lymphoid organs such as the pancreatic islets. In multiple sclerosis, EBI2 might mediate the migration of autoreactive T cells into inflamed tissues (413). The potential role of EBI2 in the pathogenesis of type 1 diabetes requires clarification in future studies.

Season of birth and environmental etiologies of beta-cell autoimmunity

This is the first study to report variation in the seasonality of birth between progressors to type 1 diabetes with variable pace of disease progression. The current findings indicate that seasonal environmental factors, such as infections or dietary factors, may affect the progression rate to clinical diabetes. The contribution of seasonal factors to the disease process is not, however, a novel idea. Exposure to infectious pathogens, such as viruses, has been proposed to trigger islet autoimmunity as early as during the fetal or neonatal period (414).

The strongest evidence for an association between type 1 diabetes and seasonal pathogens exists for enteroviruses, especially group B coxsackieviruses (258). Taking into account that enteroviral infections peak in Finland in the late summer or early fall, the observed seasonality of birth among the progressors implies that enteroviral antigen presentation during the late prenatal or early neonatal period might slow down the disease process (National Infectious Diseases Register, National Institute for Health and Welfare, Finland). This effect might be mediated through stronger protection by maternal antibodies in infants born in the fall, while infants born in the spring might encounter the corresponding antigens at an older age when they are no longer protected by maternal antibodies. In the DIPP study, the presence of maternal antiviral antibodies in cord blood attenuated the risk of coxsackievirus B1 infection-associated islet autoimmunity in offspring (264). The seasonality of birth

among patients with type 1 diabetes has been observed in numerous populations, but the findings are inconsistent, most likely due to genetic, epidemiological, and lifestyle-related differences between the various populations (415).

In this thesis, positivity for multiple biochemical autoantibodies appeared at a younger age among children born in the winter than among those born in other seasons. This raises suspicions about whether children born in the winter are exposed to environmental factors that promote progressive islet autoimmunity at a younger age than children born in other seasons. One candidate for such environmental promoters might be infections that peak during the winter such as rotavirus or norovirus infections (416). Decreased levels of vitamin D have been associated with the development of multiple autoantibodies (303). In Finland, vitamin D levels can easily diminish during the winter when the amount of sunlight is limited. Therefore, a daily vitamin D supplement is recommended for all Finnish children up to the age of 18 years starting from the age of two weeks (National Institute for Health and Welfare, Finland). However, being born in the winter did not increase the risk of type 1 diabetes relative to being born in other seasons. The association between season of birth and the development of multipositivity requires confirmation in a larger study population.

Islet cell antibodies in disease prediction

Persistent positivity for multiple biochemical autoantibodies indicates high risk for clinical diabetes and has been used as an inclusion criterion in intervention studies (149). However, the assay used for the analysis of ICA is laborious and challenging to standardize. The idea of replacing ICA with biochemical autoantibodies is therefore alluring.

We explored the dynamics of ICA in detail and examined whether removing ICA from the autoantibody screening repertoire would affect the preclinical prediction of type 1 diabetes. In contrast to previously published 5-year follow-up data of the same cohort, ICA demonstrated rather poor specificity compared with biochemical autoantibodies (7). The significantly higher specificity and PPV of multipositivity for biochemical autoantibodies relative to ICA indicate that low-titer ICA can be replaced by testing for multiple biochemical autoantibodies when screening for risk of type 1 diabetes (318, 417). However, when the threshold for ICA positivity is set at 10 JDF units, ICA predict type 1 diabetes with a high specificity (96%) and PPV (43%). The rather poor specificity of low-titer ICA indicates that most children identified only by ICA reactivity are not at any increased risk for type 1 diabetes, although the sensitivity for type 1 diabetes is relatively high (91%).

The ICA method is biological, using pancreatic tissue. Accordingly, the method detects a wide range of islet-specific antibodies and occasionally other cross-reactive antibodies (105). Because of this, the ICA method can never reach the specificity of biochemical methods, but it is more sensitive. By determining the optimal JDF level of ICA that provides the best combination of sensitivity and specificity, the ICA method can provide something more than is attainable in the four biochemical autoantibody method set and might improve the preclinical screening of type 1 diabetes.

The increasing ICA seroconversion rate towards adolescence is seen only in the case of low ICA titers, while seroconversions to high ICA titers decrease with advancing age in parallel with biochemical autoantibodies. Removing low-titer ICA from the preclinical screening plan in the general population would considerably decrease the age at seroconversion. This should be taken into account when explaining the risk of type 1 diabetes to physicians and families undergoing screening.

Among progressors to type 1 diabetes, ICA was the most stable autoantibody. Interestingly, there were no inverse seroconversions or fluctuations of ICA in progressors, while over a third of ICA-positive non-progressors reverted back to ICA negativity. Moreover, no ICA-negative children were found among multipositive individuals. This might reflect the fact that in addition to reactivity against known islet autoantigens some undiscovered autoantigens probably mediate disease-associated ICA reactivity (105).

Strengths and limitations of the study

The strength of the DIPP study is the long, regular, prospective follow-up starting from birth, allowing close collaboration between the study personnel and the participating families. The proportion of children completing the 15-year follow-up (55.8%) was acceptable considering the long duration of the follow-up. The DIPP study is carried out in Finland, which has the highest incidence of type 1 diabetes in the world, thus providing the most extensive general population-based cohort on childhood type 1 diabetes. As a downside, the high incidence of type 1 diabetes in Finland might limit the generalizability of the results. In the Finnish population, the attitude towards screening of newborn infants for the genetic risk of type 1 diabetes is generally positive. The Finnish Pediatric Diabetes Register is a national cross-sectional project that comprises information on more than 90% of pediatric diabetes cases in Finland from 2002 onwards. This database enables detection of progression to type 1 diabetes also among children who have dropped out from the preclinical DIPP study and analysis of autoantibody data obtained at the diagnosis of type 1 diabetes from all preclinically followed individuals.

Compared with other longitudinal follow-up studies on type 1 diabetes, such as Type 1 Diabetes TrialNet or TEDDY, an advantage of the DIPP study is the inclusion of both ICA and ZnT8A in the screening protocol in addition to the three other biochemical autoantibodies. Another benefit of the DIPP study is the birth cohort-based ability to define age at seroconversion in all children. In the TrialNet protocol, for instance, this is not possible in study participants recruited to the follow-up based on positivity for islet autoantibodies.

The main limitation of the DIPP study is the inclusion of only children carrying HLA risk genotypes for type 1 diabetes, which limits the generalization of the study results. However, most children (62%) diagnosed with type 1 diabetes are positive for the selected risk genotypes used in the DIPP study screening (385). It is possible that children with a positive family history for type 1 diabetes who are more genetically and environmentally prone to the disease might be more motivated to participate in the DIPP study and to complete the study follow-up than those who do not have a relative affected by type 1 diabetes. This together with the relatively high dropout rate (55.8%) might cause some selection bias in the study population.

Another limitation of the DIPP study is the ICA-based primary screening that was applied until the end of 2002, except for the selected group of the first 1006 children who were also analyzed for all four biochemical autoantibodies. Children testing positive for only biochemical autoantibodies and born before the beginning of 2003 might have been missed as autoantibody-positive since before 2003 the biochemical autoantibodies were analyzed only if the child became ICA-positive or progressed to clinical type 1 diabetes. Among the 1006 children analyzed for ICA and all four biochemical autoantibodies, 30 (3.0%) tested positive for one or more biochemical autoantibodies without ICA and would have been missed in the ICA-based screening. Moreover, the relatively low

sensitivities of the autoantibody assays imply that a proportion of autoantibody-positive children have been missed as false negatives in the screening, especially as two consecutive positive samples were required for autoantibody positivity. This may have affected the study outcomes.

Although Studies II and III are based on the whole, initially large DIPP study cohort, the number of progressors and especially rapid progressors to type 1 diabetes is modest, limiting the power of statistical analyses. The definitions of rapid and slow progression are primarily based on practical considerations, but are also partly data-driven. This might limit the generalizability of the results. However, the findings of Study II indicate that 1.5 years from seroconversion to diagnosis is a justified cut-off to distinguish rapid progressors from slower progressors. The cut-off for slow progression at 7.26 years from seroconversion provides a setting that allows evaluation of the characteristics associated with a considerably longer delay from seroconversion to diagnosis than observed for most progressors in the DIPP study.

In Study I, the number of children in the study cohort is relatively modest, which limits the power of statistical analyses, but allows testing for statistical differences. By 15.5 years, only 35 children (3.5%) had been diagnosed with type 1 diabetes. The dropout rate was relatively high, but acceptable taking into account the long duration of the follow-up. Due to the limited size of the study population, the results should be interpreted with caution. Corrections for multiple testing were not applied in the data analysis, which might limit the interpretation of the results, as positive associations may have been falsely detected by chance due to the multiple testing. Accordingly, the results should be replicated in another population. No data were available on metabolic risk factors for the study participants that might affect the risk for type 1 diabetes, which is another limitation in this thesis.

It should also be noted that although ZnT8A had been analyzed from all available samples in Study I, the information on ZnT8A was missing for some participants for the specific sample obtained at initial seroconversion. Therefore, some multipositive children at seroconversion and children with ZnT8A as the single first autoantibody at seroconversion might have been missed. This may, to some extent, have affected the observed primary autoantibody signatures among the groups of progressors, multipositive non-progressors, and single autoantibody-positive non-progressors. The associations observed between the primary autoantibodies and disease progression need to be replicated in a larger study population. However, it seems unlikely that positivity for ZnT8A during the follow-up would have remained completely undetected since ZnT8A had been analyzed from multiple samples from each individual during the long follow-up.

Considering the comparison of autoantibody titers between progressors to type 1 diabetes and their multipositive controls in Study I, the number of available matched controls was limited due to the relatively modest number of participants in the study cohort. Because of the limited selection of controls, the differences in the autoantibody titers between the two groups might have been observed by chance. Negative autoantibody titers were also included in that analysis.

In the non-HLA SNP analyses, the non-optimal data coverage and small sample size were the major limitations. SNP markers were originally analyzed for other purposes (128). As a consequence, many participants lacked data on the non-HLA SNP genotypes. The results achieved in this work require confirmation in larger study populations with more optimal data coverage. Also the association of the *FUT2* SNP with rapid disease progression must be confirmed in future studies.

Future perspectives on prediction and prevention of type 1 diabetes

As soon as a safe and effective preventive measure is available, screening for the risk of type 1 diabetes in the general population becomes justified in high-incidence countries such as Finland. Cost-effective screening at selected time points would save resources and might decrease the burden experienced by children and their families. The discoveries made in this thesis improve the preclinical estimation of type 1 diabetes risk and the timing of disease onset and can be utilized to target screening and preventive measures to optimal populations. Rapid progressors to type 1 diabetes comprise the primary target group for clinical trials and might benefit from careful clinical monitoring and early preventive measures. Targeting this group may reduce the costs of clinical trials, but as a disadvantage the generalizability of any outcomes might be limited.

As autoantibody seroconversions peak at a young age, screening in the public health context might be most effective in preschool years. Most children who progress to clinical type 1 diabetes before puberty test positive for autoantibodies before the age of 5 years (51). However, beta-cell autoimmunity may begin at an older age, and, as demonstrated in this thesis, rapid progression to type 1 diabetes occurs not exclusively in young children but also in older children approaching puberty, suggesting that additional screening points might be needed closer to puberty.

The recent staging of type 1 diabetes introduces the idea that type 1 diabetes begins as positivity for multiple biochemical autoantibodies (53). Accordingly, advanced islet autoimmunity might be seen as a disease, not only a risk for disease, and clinical interventions carried out at stage 1 or stage 2 type 1 diabetes might be considered as treatment of early disease to preserve beta-cell function rather than actual prevention.

Therefore, cost-effective screening for type 1 diabetes risk in the general population may be best achieved by screening for multiple biochemical autoantibodies. Additional monitoring might be carried out to stratify the disease risk and the pace of disease progression by using e.g. metabolic assessments. Children with only one autoantibody might be invited to follow-up visits for a few years since progression to multipositivity usually occurs within the next couple of years after initial seroconversion, but rarely thereafter (146, 175). The use of modified islet autoantigens, such as N-terminally truncated GAD, might improve our understanding of the disease pathomechanisms in the future and facilitate the identification of study subjects suitable for intervention trials (135).

To date, the treatment of type 1 diabetes is largely missing immunomodulatory therapies targeted to biological pathomechanisms. Evidence from clinical trials suggests that beta cells can be preserved to some extent at stage 2 and stage 3 (56, 71, 366, 372). However, efficacy of primary prevention, or immunotherapy at stage 1 preclinical diabetes, remains to be demonstrated. Randomized trials are challenging to carry out because any intervention administered to human populations needs to be safe, tolerable, and easily administered, with costs carefully considered in relation to benefits. In this respect, the heterogeneity in type 1 diabetes sets a challenge for conducting successful trials. For any given treatment, a therapeutic effect is seen only in a subset of treated individuals, whereas a proportion of individuals will demonstrate no effect. This implies that there are multiple pathways leading to the same phenotype and that therapeutic agents affecting one biological pathway do not reach their full potential when administered to a population comprising many individuals in whom this pathway is not active (5).

In trial design, the selection of the target population therefore plays a key role and raises the question of how to identify populations that benefit from a given treatment. Age, genetic

susceptibility, and disease severity are likely to affect outcome. The stage of disease progression sets the frame for the intensity of the applied treatment (374). As therapies targeting a single biological pathway have failed in most cases to preserve beta-cell function, the use of multifaceted therapies combining multiple mechanisms of action might improve the probability of detecting a therapeutic effect in a diverse population (371).

Disease endotypes are likely driven by distinct physiological mechanisms, but currently there is no understanding of any exact mechanisms. Factors associated with type 1 diabetes are often linked together in a way that suggests they might form pathobiological entities such as the triad of age, HLA genotype, and development of IAA vs. GADA as the first autoantibody (117, 129, 320). One important question is whether disease endotypes are driven by distinct etiological events or whether they arise in the background of shared causal mechanisms similar to all cases of type 1 diabetes such as beta-cell damage resulting from persistent viral infection (5). Age has a great impact on disease heterogeneity, but this might reflect changes in immunological maturation, metabolic function, and gene-environment interactions (diet, infections, physical and social activity) with advancing age rather than heterogeneity in causal mechanisms (325). In older children, beta-cell dysfunction rather than direct beta-cell damage might be a central mechanism in type 1 diabetes (64).

Responses to preventive therapies in clinical trials may provide important clues to disease endotypes. In the anti-CD3 teplizumab trial, for instance, persons most likely to demonstrate a therapeutic effect were characterized by the *HLA-DR4* genotype and the absence of ZnT8A (71). Disease endotypes might be identified based on the primary autoantibody, but in the public health context this would require autoantibody testing close to the time of initial seroconversion. If autoantibody screening in the general population was targeted to a few selected time points, additional biomarkers for endotyping would be required. These might include e.g. heterogeneous metabolic and cellular responses and proteomic, metabolomic, or transcriptomic patterns in prediabetic individuals. Given the multifactorial nature of type 1 diabetes, mathematical modeling of longitudinal autoantibody profiles and clusters of immune and metabolic phenotypes might facilitate the identification of complex disease endotypes and underlying biological pathways (5).

The endeavor to identify the mechanisms behind the heterogeneity in type 1 diabetes continues. A personalized approach to disease prediction and prevention would enhance the beneficial effects of a preventive treatment and decrease the potential risks and costs.

CONCLUSIONS

1. Among HLA-predisposed children recruited from the general population in Finland, conspicuous differences are present in the dynamics of islet autoantibodies during the first 15 years of life. The ICA seroconversion rate increases continuously towards puberty, but biochemical autoantibodies peak already at a young age. The primary autoantibody is characteristic of age and might reflect the etiological background driving the disease process towards clinical type 1 diabetes. In children under the age of 2 years, IAA or ZnT8A emerge commonly as the primary autoantibody, but in preschool years IA-2A and especially GADA-initiated islet autoimmunity increases. ICA becomes the prevailing autoantibody after 3 years of age, but if remaining as the only autoantibody does not confer any risk for type 1 diabetes. In young children, ZnT8A especially as the primary autoantibody might be a strong indicator of risk for type 1 diabetes.

2. The increasing ICA seroconversion rate towards adolescence is restricted to low ICA titers. High-titer ICA seroconversions decrease with advancing age in parallel with biochemical autoantibodies. The significantly higher specificity and PPV of positivity for multiple biochemical autoantibodies than for ICA indicate that low-titer ICA (<10 JDF units) can be replaced by positivity for multiple biochemical autoantibodies when screening for risk of type 1 diabetes in children. However, high ICA titers predict type 1 diabetes and in preclinical screening might provide information beyond that available in the four biochemical autoantibody method set. Excluding low-titer ICA from the screening plan in the general population would significantly affect the age at seroconversion.

3. The labile patterns of autoantibodies, especially IAA, might affect the risk for type 1 diabetes. Reversions and fluctuations of IAA are common in HLA-predisposed children, with nearly half of IAA-positive individuals returning to persistent IAA-negativity by 15 years of age. Reversions and fluctuations of ICA are also common among non-progressors, but among progressors to type 1 diabetes, ICA is the most persistent autoantibody. Children with multiple biochemical autoantibodies seem to have proceeded to a stage in the disease process at which they rarely revert to complete autoantibody negativity. Among progressors to type 1 diabetes, transient IAA positivity is associated with delayed progression from seroconversion to diagnosis, suggesting a role for the humoral immune response against insulin in the determination of the progression rate to clinical disease.

4. Rapid progressors to type 1 diabetes can be characterized among HLA-susceptible children by demographic, genetic, and immunological factors present at the initial seroconversion to autoantibody positivity. These include young age, high autoantibody titers of especially IAA, IA-2A, and ICA, multipositivity, and higher prevalence of the *HLA-DQB1*02/*03:02* genotype and the homozygous secretor genotype of the *FUT2* SNP. Rapid progression occurs not exclusively in young children under the age of 5 years, but also in children approaching puberty. Young rapid progressors are characterized by IAA positivity and high IAA titers at seroconversion, and older rapid progressors by GADA positivity and high GADA titers. These

differences might reflect the IAA vs. GADA-driven endotypes of type 1 diabetes and suggest that mechanisms behind rapid progression might be independent of the etiological triggers of the disease process. The association of the homozygous *FUT2* secretor genotype with rapid progression suggests that a higher amount of soluble ABO histo-blood group antigens in bodily secretions and on the intestinal mucosa might promote events that contribute to rapid progression to type 1 diabetes. Rapid progressors to type 1 diabetes comprise the primary target group for clinical prevention trials. To cover both young and prepubertal populations of rapid progressors, future screening for risk of type 1 diabetes in the public health context might be targeted to a few selected age points, e.g. one at preschool age and another closer to puberty.

5. Slow progressors to type 1 diabetes may be distinguished from other progressors by immunological characteristics present at seroconversion, including lower titers of IAA and ICA and lower frequency of positivity for IA-2A and multiple biochemical autoantibodies. Progression from single to multiple autoantibody positivity is delayed among slow progressors. Season of birth contributes to the progression rate from seroconversion to clinical diabetes. Disease progression is accelerated among children born in the spring and is slower among children born in the fall. The impact of season of birth on the rate of functional beta-cell loss indicates that seasonal environmental factors contribute substantially to the pathogenesis of type 1 diabetes. The timing of exposure to certain environmental factors in relation to a child's immunological development might be a major determinant in setting the pace of disease progression.

6. No associations were found between the non-HLA SNPs analyzed and slow disease progression after corrections for multiple testing. As expected, the predisposing SNPs in the *PTPN22*, *INS* (rs689), and *PHTF1* genes showed associations with overall progression to type 1 diabetes. Also the predisposing *EBI2*/*GPR183* SNP tended to associate with progression to type 1 diabetes. EBI2 might be of relevance in future pathomechanistic studies of type 1 diabetes considering its pivotal role in humoral immune responses and its potential contribution to the development of central tolerance. However, this finding must be confirmed in a larger study population with improved data coverage.

In summary, several demographic, genetic, and autoantibody characteristics were found to be associated with progression rate to type 1 diabetes in this work, but we observed no unambiguous characteristics specifically predictive for slow disease progression. The unique variations in the dynamics of islet autoantibodies support the idea of heterogeneity in the disease process. Apart from genetic and etiological elements, age and the stage of immunological maturation contribute substantially to the characteristics of islet autoimmunity. These findings provide an important framework for understanding the heterogeneous nature of type 1 diabetes and might be utilized in future trial design to personalize the efforts to delay or prevent the onset of type 1 diabetes. Further investigation of the age-specific features of beta-cell autoimmunity might provide mechanistic clues to the driving events of the disease process. The observed associations between the season of birth and positivity for multiple autoantibodies, the primary autoantibodies and disease progression, and the *FUT2* SNP and rapid progression require confirmation in future studies.

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